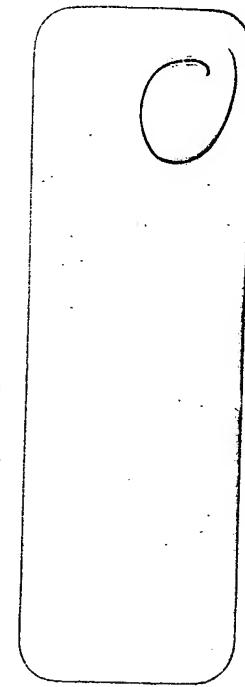


P.O. BOX 1450
ALEXANDRIA, VA 22313-1450
IF UNDELIVERABLE RETURN IN TEN DAYS

OFFICIAL BUSINESS

AN EQUAL OPPORTUNITY EMPLOYER



KNGE201 9411120441804 14 09/12/05
FORWARD TIME EXP RTN TO SEND
KNOBBE MARTENS OLSON & BEAR
1 SAN SIMEON ST PL 35
SAN FRANCISCO CA 94104-4436
RETURN TO SENDER
||||||||||||||||||||||||||||||||



BEST AVAILABLE COPY



UNITED STATES PATENT AND TRADEMARK OFFICE

1649
JW
UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/036,063	12/26/2001	Audrey Goddard	P3030R1C6	4326
7590	09/01/2005			EXAMINER KOLKER, DANIEL E
Ginger R. Dreger Knobbe Martens Olson & Bear Suite 1150 201 California Street San Francisco, CA 94111			ART UNIT 1649	PAPER NUMBER
DATE MAILED: 09/01/2005				

Please find below and/or attached an Office communication concerning this application or proceeding.

RECEIVED
OIPE/IAP

SEP 21 2005

Office Action Summary	Application No.	Applicant(s)	
	10/036,063	GODDARD ET AL.	
Examiner	Art Unit		
Daniel Kolker	1649		

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1) Responsive to communication(s) filed on 20 June 2005.

2a) This action is FINAL. 2b) This action is non-final.

3) Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4) Claim(s) 22-26 and 28-30 is/are pending in the application.
4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5) Claim(s) _____ is/are allowed.

6) Claim(s) 22-26 and 28-30 is/are rejected.

7) Claim(s) _____ is/are objected to.

8) Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9) The specification is objected to by the Examiner.

10) The drawing(s) filed on _____ is/are: a) accepted or b) objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11) The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12) Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a) All b) Some * c) None of:

1. Certified copies of the priority documents have been received.
2. Certified copies of the priority documents have been received in Application No. ____.
3. Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) Notice of References Cited (PTO-892) 4) Interview Summary (PTO-413)
2) Notice of Draftsperson's Patent Drawing Review (PTO-948) Paper No(s)/Mail Date. ____ .
3) Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) 5) Notice of Informal Patent Application (PTO-152)
Paper No(s)/Mail Date 6/20/05. 6) Other: ____ .

DETAILED ACTION

1. The Art Unit location of your application in the USPTO has changed. To aid in correlating any papers for this application, all further correspondence regarding this application should be directed to Art Unit 1649.
2. Applicant's remarks, amendments, and declarations filed 20 June 2005 have been entered. Claim 27 has been cancelled, new claims 28 – 30 have been added. Claims 22 – 26 and 28 – 30 are pending and under examination.
3. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.
4. The deletion of inventors under 37 CFR 1.48(b) is acknowledged.

Information Disclosure Statement

5. The information disclosure statement filed 20 June 2005 has been considered. The BLAST results indicate that applicants are aware of nucleic acids and proteins with identity or homology to the one claimed herein. However the results cannot be considered because there is no alignment provided, nor is there an indication of the percent identity between the claimed sequence and the reference sequences. Applicant states on p. 5 of the remarks that the newly-submitted documents include references to specific accession numbers and sequences. Applicant is advised that the BLAST results submitted appear to be a list of sequences which match, but do not provide either alignments or indications of how the sequences are related to the instantly-claimed peptides. Therefore the examiner cannot determine if the sequence accession numbers submitted by applicant constitute prior art. Furthermore the search results submitted appear to be the results are not publicly available documents. Applicant is directed to MPEP 609 and 37 CFR 1.97 and 1.98.

Withdrawn Objections and Rejections

6. The following objections or rejections made in the previous office action are withdrawn:
The objections to the specification. Applicant has deleted the hyperlinks and changed the title.

The rejection of claims 22 - 27 under 35 USC 112, second paragraph. Applicant has cancelled the claim.

Rejections Maintained***Claim Rejections - 35 USC §§ 101 and 112***

7. Claims 22 – 26 and 28 – 30 are rejected under 35 U.S.C. 101 because the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility.

The rejection is maintained for the reasons made of record in the previous office action and reiterated below.

The claims are drawn to antibodies which bind to SEQ ID NO:57, also called PRO4380. There is no asserted utility for antibodies which bind to PRO4380 independent from their utilities in purifying, detecting, or binding to PRO4380. For example, there is no contemplation of use of anti-PRO4380 antibodies as therapeutics in treating a specific disease. There is no disclosure of informative data on how purifying or detecting PRO4380 would be useful. An antibody that binds to a protein with a specific and substantial utility would itself be useful, as it could be used to purify that protein. An antibody could also be useful if it could be used to distinguish between patients with a disease and those free of disease, for example. However, in the instant case, the antibody has not been shown to be useful in the detection or treatment of disease or conditions, or in the purification of a protein that has utility. The utility of the antibodies hinges on whether or not PRO994 itself is useful.

The specification asserts that PRO4380 has two specific utilities, as it came up positive in two assays, however neither utility is substantial.

Applicant did not address the examiner's arguments that the first assay, Example 37 (page 166), drawn to compounds which test positive as either stimulators or inhibitors of glucose or FFA uptake, does not constitute a reasonable test for useful compounds. This assay is deemed to lack utility for the reasons made of record in the previous office action.

The data presented in Example 41 (p. 168 – 169) of the specification indicate that PRO4380 was positive in the Mouse Kidney Mesangial Cell Proliferation Assay. It is acknowledged that proliferation of mammalian kidney mesangial cells is useful, as Schocklmann (1999. Kidney International 56:1199-1207) teaches that such proliferation is necessary after injury or damage to the kidney. However, the threshold used in determining whether a particular PRO molecule counts as "positive" in this assay would not be considered reasonable by one of skill in the art. The specification discloses (p. 169, lines 1 – 2) that positives in this assay include anything which is at least 15% over the control reading. The post-filing

publication by Rovin et al. (2002. *Kidney International* 61:1293-1302) indicates that a 21% increase in human mesangial cell proliferation is not statistically significant (see particularly p. 1296, lines 3 – 6).

On page 6 of the remarks applicant refers to the utility guidelines on specific, substantial, and credible utilities. The claims were not rejected for lack of a specific or credible utility, thus arguments related to those (i.e page 6, points (1) and (3) in the final paragraph) are not germane. Particularly, the citation of MPEP 2107 II (B)(1)(ii), drawn to credible utilities is not on point as no rejection for lack of credibility was made.

On p. 7 of the remarks, applicant argues that utility need not be proven, that a reasonable correlation between the evidence and the asserted utility is sufficient, and that the asserted utility should be accepted if it is more likely than not true. Applicant cites *In re Langer*, *In re Jolles*, *In re Irons*, *In re Sichert*, *Raytheon v. Roper*, and *In re Oetiker* as supporting this argument. Applicant's arguments have been fully considered but are not persuasive.

In *In re Langer*, the court ruled the Patent Office cannot require clinical testing in humans to rebut a *prima facie* case for lack of utility. In the instant case, the Office has not made such a requirement. Furthermore the Langer court ruled that "Assuming that sufficient reason to question the statement of utility and its scope does exist, a rejection for lack of utility under § 101 will be proper on that basis; such a rejection can be overcome by suitable proofs indicating that the statement of utility and its scope as found in the specification are true." In the instant case there is in fact sufficient reason to question the statement of utility. The reference by Rovin cited in the previous office action indicates that the 15% threshold used by applicant is not reasonable. Therefore one of skill in the art would have reason to doubt the asserted utility.

In *In re Jolles*, the issue was whether data from an art-recognized animal model could be considered predictive of results in humans. That is not an issue in the instant case, as the examiner indicated in the paragraph spanning pp. 5 – 6 of the previous office action that proliferation of mammalian kidney mesangial cells would be useful, as it is an important part of the repair process after injury (see Schocklmann, particularly pp. 1199 - 1200 for more detailed discussion). But since the threshold used by applicant was 71% of a change shown not to be statistically significant, one of skill in the art would conclude that the PRO4380 does not induce any more mesangial cells than are induced under control conditions.

The citation of *In re Irons* is also not relevant to the instant case. In *Irons*, evidence was submitted that indicated that the drug had been administered to 888 patients and that

Art Unit: 1649

statistically significant results were obtained showing an improvement in arthritic conditions. In the instant case, no such evidence has been submitted. In the instant case, the claimed product has not been administered to patients. Furthermore, there is no evidence of record indicating a statistically significant result in vitro.

The *Sichert* court ruled that blind comparative studies of the claimed compositions, which showed that the compositions were effective in relieving lymphatic congestion (as narrowly defined), were sufficient to establish utility of said compositions under 35 USC § 101. In the instant case, applicant has not shown any such studies, and therefore because the fact pattern is sufficiently different the *Sichert* case is not germane.

In *Raytheon v. Roper*, utility was found by the Federal Circuit when a lack of utility had been found by a lower court. This was due not to the sufficiency of the evidence presented, but rather because the Federal Circuit ruled that the claims in question had been interpreted erroneously. In the instant case, there does not appear to be a question as to how the pending claims are being interpreted.

It is not immediately apparent why applicant has cited *In re Oetiker* in arguments related to the utility under 35 USC § 101, as the *Oetiker* case dealt not with utility but with obviousness under 35 USC § 103. No claims have been rejected under § 103 in the instant case.

The examiner acknowledges that the ability to induce mesangial cell proliferation is specific. However, the assay used by applicant and reported in Example 41 beginning on p. 168 of the specification would not allow a skilled artisan to conclude that it is more likely than not that the asserted utility is true and therefore the asserted utility is not substantial.

On p. 8 of the remarks applicant discusses the examiner's interpretation of the results from Rovin. The examiner and applicant appear to agree on this point. Rovin clearly stated that the data point, a 21% increase, did not represent a significant difference due to the large degree of variability inherent in this assay. Because of the large degree of error, a 21% increase is not significant. Stated another way, one of skill in the art would recognize that it is *improper* to conclude that the two samples (control and 5 uM ciglitazone) are drawn from different populations.

Significance does not mean, as applicant asserts on p. 8 of the remarks, that there is not an overlap of standard deviations or errors in the data set. Rather, statistical significance is a determination, based on mathematic procedures, that the observed difference between samples has a less than 5% chance of occurring by random accident (see attached definition from the

Art Unit: 1649

On-line Medical Dictionary, accessed 22 July 2005). Applicant argues that Rovin's report of a 21% non-significant difference indicates that the statistical error in their measurement overlaps with the statistical error in the control set, and that "this does not mean that an increase of proliferation of 21% is not scientifically important or significant, but means that Rovin's particular measurement of 21% may be incorrect due to the amount of error for that data point." This is not the way significance is understood in the art. Significance is an inference. When a result reaches statistical significance, it is proper to infer that the two samples are drawn from separate populations. When the result is not significant, the appropriate inference is not that the particular value is subject to error, but rather that it is not possible to tell if the two samples were drawn from separate populations. Applicant is directed to the attached text from the chapter by Freund et al. (2003. Statistical Methods, Second Edition, pp. 117 – 138), particularly the definition of "significance level" on p. 126 and the definition of "p value" on p. 133 for a more complete understanding of the way statistics are used in scientific papers. In the instant case, Rovin et al. used analyses of variance, followed by post-hoc Bonferroni-corrected pairwise comparisons (see p. 1295, second column "Statistical Analysis") rather than the standard argued by applicant. The teachings of Rovin indicate that this assay has so much variability that even if a 21% difference is detected when 12 experimental and 12 control samples are provided (see legend for Figure 2 which indicates that "[e]ach point represents the mean of at least 3 individual experiments done in quadruplicate".

On p. 9 of the remarks, applicant points out that Rovin found an 18% increase was significant. Applicant again asserts his own definition of significance (p. 12, second paragraph) which contradicts that provided on p. 1295 of Rovin. It is noted that this level is still greater than the changes reported in the specification, where only a 15% increase is considered important. However, taken with the finding that a 21% increase in this assay is not significant, this finding further supports the examiner's point that knowing the variability associated with the measurements is crucial to determining whether or not the artisan will conclude that samples are drawn from different populations. In the instant case, the specification does not disclose the variability in the sample, so a skilled artisan would not reasonably conclude that PRO4380 induces mesangial cell proliferation. Neither PRO4380 nor antibodies which bind to it are useful. Thus the rejection under 35 USC § 101 stands.

8. Claims 22 – 26 and 28 – 30 are also rejected under 35 U.S.C. 112, first paragraph. Specifically, since the claimed invention is not supported by either a specific and substantial

asserted utility or a well established utility for the reasons set forth above, one skilled in the art clearly would not know how to use the claimed invention.

Claim Rejections - 35 USC § 102

9. Claims 22 – 25 and new claims 28 – 29 are rejected under 35 U.S.C. 102(a) as being anticipated by Ruben et al. (WO 99/58660, published 18 November 1999) as evidenced by Harlow et al. (1988. Antibodies: A Laboratory Manual) for the reasons made of record in the previous office action and reiterated herein. Ruben et al. teach specific preferred epitopes along the entire length of the protein which can be used for the production of antibodies (see p. 49, lines 17 – 20). Ruben et al. further teach that their antibodies include monoclonal antibodies, Fab and F(ab')2 fragments, chimeric, single-chain, and humanized antibodies (see paragraph spanning pp. 196 – 197). As detailed in the previous office action, the antibodies from Ruben would be expected to bind to SEQ ID NO:57, and Harlow provides evidence of such.

On page 10 of the remarks applicant refers to the declaration by Goddard et al. as providing support for the argument that the 102 rejection should be withdrawn because applicant possessed the claimed invention before the reference was published. The declaration filed on 20 June 2005 under 37 CFR 1.131 has been considered but is ineffective to overcome the Ruben reference. The evidence submitted is insufficient to establish a reduction to practice of the invention in this country or a NAFTA or WTO member country prior to the effective date of the Ruben reference.

Paragraph 5 of the declaration by Goddard et al. indicates that applicant was in possession of the protein with SEQ ID NO:57 before the date of the Ruben reference. Paragraph 8 of the declaration indicates that applicant reduced the protein to practice prior to the date of the Ruben reference. The instantly-claimed invention is an antibody, not a protein. The declaration submitted does not provide evidence of reducing the antibody to practice before the date of the Ruben reference. Therefore the reference is not sufficient to overcome the rejection of claims 22 – 25.

Claim Rejections - 35 USC § 103

10. Claims 22, 25, 26, and 30 are rejected under 35 U.S.C. 103(a) as being unpatentable over Ruben in view of Holmes et al. (Current Protocols in Immunology, cited in previous office

Art Unit: 1649

action). Ruben et al. teach antibodies which bind to SEQ ID NO:57. Ruben et al. do not teach a labeled antibody. Holmes et al. teach conjugation of multiple labels (FITC, biotin, Texas Red, and phycobiliproteins) to antibodies for detection. It would have been obvious to one of ordinary skill in the art to label either the antibodies or the fragments from Ruben, as taught by Holmes, with a reasonable expectation of success. The motivation to do so would be to detect the antibodies or fragments, as Holmes teaches that labeling is useful for quantification of the antigen to which the antibody binds, and can be done with antibodies in general.

On page 12 of the remarks applicant refers to the declaration by Goddard et al. as providing support for the argument that the 103 rejection should be withdrawn because applicant possessed the claimed invention before the reference was published. The declaration filed on 20 June 2005 under 37 CFR 1.131 has been considered but is ineffective to overcome the Ruben reference. The evidence submitted is insufficient to establish a reduction to practice of the invention in this country or a NAFTA or WTO member country prior to the effective date of the Ruben reference.

Paragraph 5 of the declaration by Goddard et al. indicates that applicant was in possession of the protein with SEQ ID NO:57 before the date of the Ruben reference. Paragraph 8 of the declaration indicates that applicant reduced the protein to practice prior to the date of the Ruben reference. The instantly-claimed invention is an antibody, not a protein. The declaration submitted does not provide evidence of reducing the antibody to practice before the date of the Ruben reference. Therefore the declaration is not sufficient to overcome the rejection of claims 22 and 25, as explained in the rejection under 35 USC 102 above. The declaration is also not sufficient to overcome the rejection of claims 26 and 30, as it would still be obvious to label the antibodies and fragments. Applicant did not traverse the examiner's rejection of obviousness and since the declaration is not sufficient to overcome the reference the rejection stands.

Rejections Necessitated by Amendment

Claim Rejections - 35 USC § 102

(a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for a patent.

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Art Unit: 1649

11. Claims 25, 28, and 29 are rejected under 35 U.S.C. 102(a) as being anticipated by Ruben et al. (WO 99/58660, published 18 November 1999) as evidenced by Harlow et al. (1988. Antibodies: A Laboratory Manual). The reasons why claim stands rejected is explained in detail above in the section under Rejections Maintained. Briefly, Ruben teaches a sequence very similar to instantly-claimed SEQ ID NO:57, and Harlow provides evidence that the antibody from Ruben would be expected to bind to applicant's SEQ ID NO:57. New claims 28 – 29 are also rejected because Ruben defines "monoclonal antibody" as including antibody fragments, and also includes humanized antibodies within the scope of this definition, thereby meeting the limitation of claims 28 – 29 (see Ruben pp. 196 – 197).

New Rejections and Objections

Oath/Declaration

12. The oath or declaration is defective. A new oath or declaration in compliance with 37 CFR 1.67(a) identifying this application by application number and filing date is required. See MPEP §§ 602.01 and 602.02.

The oath or declaration is defective because:

Non-initialed and/or non-dated alterations have been made to the oath or declaration, particularly the changes to citizenship by Zhang and to address by Eaton. See 37 CFR 1.52(c).

Claim Rejections - 35 USC § 102

13. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

14. Claims 22 – 25 and 28 – 29 are rejected under 35 U.S.C. 102(e) as anticipated by Ruben et al. (U.S. Patent application publication 2003/0100051, published 29 May 2003, filed 10 September 2001, claiming priority to applications filed 28 June 2001, 10 November 1999, 6

May 1999, and claiming benefit of provisional applications filed 11 September 2000, 18 May 1998, and 12 May 1998), as evidenced by Harlow et al. (cited in previous office action).

Ruben et al. teach SEQ ID NOs:137, 139, and 242, each of which are 97.0% identical to applicant's SEQ ID NO:57 (see attached alignments). Ruben's sequences are identical to applicant's SEQ ID NO:57 from residues 16 – 507 (using applicant's residue numbers). Ruben teaches antibodies to these sequences, including both monoclonal and humanized antibodies as well as Fab fragments, F(ab') fragments, and fragments produced by a Fab expression library (see page 116, paragraph 0796). Although the polypeptide sequence of Ruben et al. is not identical to that of SEQ ID NO:57, the two are so close that it is expected that an antibody raised against either one would recognize the other. The teachings of Harlow et al. are particularly informative. Page 76 of Harlow indicates that long peptides, including the hydrophilic regions, are likely to produce antibodies and that sequences as short as six amino acid residues can be immunogenic. Clearly the high degree of identity between the two peptide sequences, and the fact that the hydrophobic region identified by applicant as the transmembrane domain is identical in both, indicates that the antibodies produced by Ruben et al. will recognize the polypeptide of SEQ ID NO:57. The prior art teachings of Ruben et al. therefore meet the limitations of claims 22 – 25 and 28 - 29.

It is acknowledged that applicant's declaration under 37 CFR 1.131 is sufficient to indicate that he was in possession of the claimed material prior to 18 November 1999. However the publication by Ruben cited herein claims benefit of provisional applications filed 12 May 1998 and 18 May 1998. These are the same applications cited on the face of WO 99/58660, which was the basis of the rejection under 35 USC 102 (a) in the previous office action.

Claim Rejections - 35 USC § 103

15. Claims 22, 25, 26, and 30 are rejected under 35 U.S.C. 103(a) as being unpatentable over Ruben et al. (U.S. Patent application publication 2003/0100051, published 29 May 2003, filed 10 September 2001, claiming priority to applications filed 28 June 2001, 10 November 1999, 6 May 1999, and claiming benefit of provisional applications filed 11 September 2000, 18 May 1998, and 12 May 1998) in view of Holmes et al (1995. Current Protocols in Immunology, pp. 5.3.5 – 5.3.8). Ruben et al. teach antibodies which bind to SEQ ID NO:57 and fragments of the antibodies, as explained above. Ruben et al. do not teach a labeled antibody. Holmes et al. teach conjugation of multiple labels (FITC, biotin, Texas Red, and phycobiliproteins) to

Art Unit: 1649

antibodies for detection. It would have been obvious to one of ordinary skill in the art to label the antibodies or antibody fragments of Ruben et al. for purposes of detecting PRO4380, using one of the protocols provided by Holmes et al., with a reasonable expectation of success. A motivation for doing so would be to label a cell or cells that express PRO4380, and Holmes teaches preferred methods for labeling as recognized by the skilled artisan.

Conclusion

16. No claim is allowed. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Daniel Kolker whose telephone number is (571) 272-3181. The examiner can normally be reached on Mon - Fri 8:30AM - 5:00PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Janet Andres can be reached on (571) 272-0867. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Daniel E. Kolker, Ph.D.

August 16, 2005

Sharon Turner
SHARON TURNER, PH.D.
PRIMARY EXAMINER
818-05

Notice of References Cited		Application/Control No.	Applicant(s)/Patent Under Reexamination GODDARD ET AL.	
		Examiner Daniel Kolker	Art Unit 1649	Page 1 of 2

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	On-line Medical Dictionary, definition for "significance" accessed 22 July 2005
	V	Freund et al. 2003. Statistical Methods, Second Edition, pp. 117 – 138.
	W	Alberts et al., Molecular Biology of the Cell, p. 1216-1217
*	X	Ruben et al. U.S. Patent application publication 2003/0100051, published 29 May 2003.

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Notice of References Cited		Application/Control No.	Applicant(s)/Patent Under Reexamination GODDARD ET AL.	
		Examiner	Art Unit	Page 2 of 2
		Daniel Kolker	1649	

U.S. PATENT DOCUMENTS

*	Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
A	US-			
B	US-			
C	US-			
D	US-			
E	US-			
F	US-			
G	US-			
H	US-			
I	US-			
J	US-			
K	US-			
L	US-			
M	US-			

FOREIGN PATENT DOCUMENTS

*	Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
N					
O					
P					
Q					
R					
S					
T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Schocklmann 1999. Kidney International 56:1199-1207
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

INFORMATION DISCLOSURE STATEMENT
BY APPLICANT

(USE SEVERAL SHEETS IF NECESSARY)

JUN 24 2004

PTO-1449

ATTY. DOCKET NO.
GNE.3030R1C6APPLICATION NO.
10/036,063APPLICANT
Desnoyers, et al.FILING DATE
December 26, 2001GROUP
1848

U.S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE (IF APPROPRIATE)

FOREIGN PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION	
						YES	NO
JK	1 WO 96/14331	5/17/1996	PCT	PCT			
JK	2 WO 97/25427	7/17/1997	PCT	PCT			
JK	3 WO 99/06551	2/11/1999	PCT	PCT			
JK	4 WO 99/55865	11/04/1999	PCT	PCT			

OTHER DOCUMENTS (INCLUDING AUTHOR, TITLE, DATE, PERTINENT PAGES, ETC.)

JK	5 Tashiro, et al. "Signal Sequence Trap: A Cloning Strategy for Secreted Proteins and Type I Membrane Proteins." <i>Science</i> . 26: 600-603 (July 30, 1993).

S:\DOCS\IMTM\IMTM-6290.DOC sma060804

EXAMINER <i>Janet G. Johnson</i>	DATE CONSIDERED <i>8/19/05</i>
*EXAMINER: INITIAL IF CITATION CONSIDERED, WHETHER OR NOT CITATION IS IN CONFORMANCE WITH MPEP 609; DRAW LINE THROUGH CITATION IF NOT IN CONFORMANCE AND NOT CONSIDERED. INCLUDE COPY OF THIS FORM WITH NEXT COMMUNICATION TO APPLICANT.	

BEST AVAILABLE COPY

GNE.3030R1C6

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE



Applicant	:	Goddard et al. (as amended)
Appl. No.	:	10/036,063
Filed	:	December 26, 2001
For	:	ANTIBODIES TO POLYPEPTIDES THAT INDUCE CELL PROLIFERATION (as amended)
Examiner	:	Kolker, Daniel E.
Group Art Unit	:	1646

DECLARATION UNDER 37 CFR §1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

We declare and state as follows:

*DK
considered
8/11/05*

1. We are the inventors of the invention claimed in the above-captioned patent application.
2. During the time period in which we participated in the events and activities described herein, we were employed by Genentech, Inc., the assignee of the above-captioned application.
3. All of the events and activities described herein were performed by us personally, or by others at our direction as part of our duties as employees of Genentech, Inc.
4. The invention claimed in the above-captioned patent application was conceived and reduced to practice in the United States prior to November 18, 1999 as described below.
5. Prior to November 18, 1999, we conceived of the invention claimed in the above-captioned patent application. This is demonstrated by the attached sequence printout (Exhibit A), which was generated prior to November 18, 1999, and which shows the complete sequence of the nucleic acid having the sequence of SEQ ID NO: 56. The attached printout also shows the complete sequence of the polypeptide which has the sequence of SEQ ID NO: 57. As evidenced by the sequence printout, we were in possession of the complete nucleic acid and amino acid sequences prior to November 18, 1999.
6. The date deleted from Exhibit A is prior to November 18, 1999. This date was redacted pursuant to M.P.E.P. § 715.07. The date that remains is the date the report was printed, April 28, 2005.
7. After these initial experiments, we diligently reduced the claimed subject matter to practice by working to express and purify the encoded polypeptide and to run it systematically through many assays. The cDNA was deposited with the American Type Culture Collection (ATCC) on April 20, 1999 and assigned ATCC no. 203948. The protein of interest was assigned a "protein inventory

[Home](#)[Help](#)[Subjects](#)[Feedback](#)[Random](#)[Search OMD](#)

significance

An analytical evaluation of the results of a comparative trial or survey. Data yielding a difference in outcome depending on treatment or environmental factors are considered statistically significant if various mathematical procedures indicate there is less than a one in twenty (five percent) chance that the same results would occur through random accident. (In statistical terms this is expressed as $p < .05$ -or, the p-value is less than 0.05.)

(09 Oct 1997)

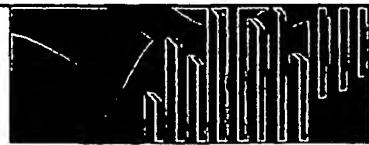
Previous: [sign blindness](#), [signet ring](#), [signet-ring cell carcinoma](#), [signet ring cells](#)

Next: [significant](#), [significative](#), [sign language](#), [sign of elongation](#)

Published at the Centre for Cancer Education, [University of Newcastle upon Tyne](#)
© Copyright 1997-2005 - The CancerWEB Project. All Rights Reserved.

BEST AVAILABLE COPY

Statistical Methods



Second Edition

Rudolf J. Freund
Texas A&M University

William J. Wilson
University of North Florida

04-30-04A09:40 RCVD



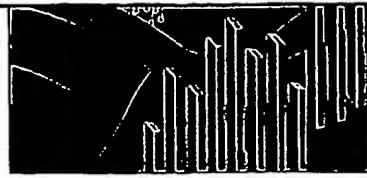
ACADEMIC PRESS

An imprint of Elsevier Science

Amsterdam Boston London New York Oxford Paris
San Diego San Francisco Singapore Sydney Tokyo

is is close to,
result of 600

Chapter 3



Principles of Inference

EXAMPLE 3.1

Is Office Rent More Expensive in Atlanta? A businessman in Atlanta is considering moving to Jacksonville, Florida, to reduce the office rental costs for his company. In the October 1990 issue of the *Jacksonville Journal*, the mean cost of leasing office space for all downtown buildings in Jacksonville was quoted as being \$12.61 per square foot with a standard deviation of \$4.50. To compare costs with those in Atlanta, the businessman sampled 36 office buildings in Atlanta and found a mean leasing cost of \$13.55 per square foot. Does this mean that leasing office space in Atlanta is really higher? Should the businessman consider moving to Jacksonville to save money on rent (assuming other factors equal)? This chapter presents methodology that can be used to help answer this question. This problem will be solved in Section 3.2. ■

3.1 Introduction

As we have repeatedly noted, one of the primary objectives of a statistical analysis is to use data from a sample to make inferences about the population from which the sample was drawn. In this chapter we present the basic procedures for making such inferences.

As we will see, the sampling distributions discussed in Chapter 2 play a pivotal role in statistical inference. Because inference on an unknown population parameter is usually based solely on a statistic computed from a single sample, we rely on these distributions to determine how reliable this inference is. That is, a statistical inference is composed of two parts:

1. a *statement* about the value of that parameter, and
2. a measure of the *reliability* of that statement, usually expressed as a probability.

Traditionally statistical inference is done with one of two different but related objectives in mind.

1. We conduct tests of hypotheses, in which we hypothesize that one or more parameters have some specific values or relationships, and make our decision about the parameter(s) based on one or more sample statistic. In this type of inference, the reliability of the decision is the probability that the decision is incorrect.
2. We estimate one or more parameters using sample statistics. This estimation is usually done in the form of an interval, and the reliability of this inference is expressed as the level of confidence we have in the interval.

We usually refer to an incorrect decision in a hypothesis test as "making an error" of one kind or another. Making an error in a statistical inference is not the same as making a mistake; the term simply recognizes the fact that the possibility of making an incorrect inference is an inescapable fact of statistical inference. The best we can do is to try to evaluate the reliability of our inference. Fortunately, if the data used to perform a statistical inference are a random sample, we can use sampling distributions to calculate the probability of making an error and therefore quantify the reliability of our inference.

In this chapter we present the basic principles for making these inferences and see how they are related. As you go through this and the next two chapters, you will note that hypothesis testing is presented before estimation. The reason for this is that it is somewhat easier to introduce them in this order, and since they are closely related, once the concept of the hypothesis test is understood, the estimation principles are easily grasped. We want to emphasize that both are equally important and each should be used where appropriate. To avoid extraneous issues, in this chapter we use two extremely simple (and not very interesting) examples that have little practical application. Once we have learned these principles, we can apply them to more interesting and useful applications. That is the subject of the remainder of this book.

3.2 Hypothesis Testing

A hypothesis usually results from speculation concerning observed behavior, natural phenomena, or established theory. If the hypothesis is stated in terms of population parameters such as the mean and variance, the hypothesis is called a **statistical hypothesis**. Data from a sample (which may be an experiment) are used to test the validity of the hypothesis. A procedure that enables us to agree or disagree with the statistical hypothesis using data from a sample is called a **test of the hypothesis**. Some examples of hypothesis tests are:

- A consumer-testing organization determining whether a type of appliance is of standard quality (say, an average lifetime of a prescribed length) would base their test on the examination of a sample of prototypes of the

different but

to one or more take our deci-
stic. In this
bility that the

his estimation
this inference
1.

is "making an
ference is not
fact that the
fact of statis-
tability of our
ference are a
he probability
ference.

use inferences
ext two chap-
timation. The
in this order,
thesis test is
int to empha-
where appro-
vo extremely
actical appli-
hem to more
tander of this

ved behavior,
ated in terms
hypothesis is
y be an exper-
ture that en-
g data from a
pothesis tests

of appliance
ribed length)
otypes of the

appliance. The result of the test may be that the appliance is not of acceptable quality and the organization will recommend against its purchase.

- A test of the effect of a diet pill on weight loss would be based on observed weight losses of a sample of healthy adults. If the test concludes the pill is effective, the manufacturer can safely advertise to that effect.
- To determine whether a teaching procedure enhances student performance, a sample of students would be tested before and after exposure to the procedure and the differences in test scores subjected to a statistical hypothesis test. If the test concludes that the method is not effective, it will not be used.

General Considerations

To illustrate the general principles of hypothesis testing, consider the following two simple examples:

EXAMPLE 3.2

There are two identically appearing bowls of jelly beans. Bowl 1 contains 60 red and 40 black jelly beans, and bowl 2 contains 40 red and 60 black jelly beans. Therefore, the proportion of red jelly beans, p , for the two bowls are

$$\text{Bowl 1: } p = 0.6$$

$$\text{Bowl 2: } p = 0.4.$$

One of the bowls is sitting on the table, but you do not know which one it is (you cannot see inside it). You suspect that it is bowl 2, but you are not sure. To test your hypothesis that bowl 2 is on the table you sample five jelly beans.¹ The data from this sample, specifically the number of red jelly beans, is the sample statistic that will be used to test the hypothesis that bowl 2 is on the table. That is, based on this sample, you will decide whether bowl 2 is the one on the table. ■

EXAMPLE 3.3

A company that packages salted peanuts in 8-oz. jars is interested in maintaining control on the amount of peanuts put in jars by one of its machines. Control is defined as averaging 8 oz. per jar and not consistently over- or underfilling the jars. To monitor this control, a sample of 16 jars is taken from the line at random time intervals and their contents weighed. The mean weight of peanuts in these 16 jars will be used to test the hypothesis that the machine is indeed working properly. If it is deemed not to be doing so, a costly adjustment will be needed.² ■

These two examples will be used to illustrate the procedures presented in this chapter.

¹To make the necessary probability calculations easier, you replace each jelly bean before selecting a new one; this is called sampling with replacement and allows the use of the binomial probability distribution presented in Section 2.3.

²Note the difference between this problem and Example 2.13, the control chart example. In this case, a decision to adjust the machine is to be made on one sample only, while in Example 2.13 it is made by an examination of its performance over time.

The Hypotheses

Statistical hypothesis testing starts by making a set of two statements about the parameter or parameters in question. These are usually in the form of simple mathematical relationships involving the parameters. The two statements are exclusive and exhaustive, which means that one or the other statement must be true, but they cannot both be true. The first statement is called the *null hypothesis* and is denoted by H_0 , and the second is called the *alternative hypothesis* and is denoted by H_1 .

DEFINITION 3.1

The **null hypothesis** is a statement about the values of one or more parameters. This hypothesis represents the status quo and is usually not rejected unless the sample results strongly imply that it is false.

For Example 3.2, the null hypothesis is

Bowl 2 is on the table.

In bowl 2, since 40 of the 100 jelly beans are red, the statistical hypothesis is stated in terms of a population parameter, p = the proportion of red jelly beans in bowl 2. Thus the null hypothesis is

$$H_0: p = 0.4.$$

DEFINITION 3.2

The **alternative hypothesis** is a statement that contradicts the null hypothesis. This hypothesis is declared to be accepted if the null hypothesis is rejected. The alternative hypothesis is often called the research hypothesis because it usually implies that some action is to be performed, some money spent, or some established theory overturned.

In Example 3.2 the alternative hypothesis is

Bowl 1 is on the table,

for which the statistical hypothesis is

$$H_1: p = 0.6,$$

since 60 of the 100 jelly beans in bowl 1 are red. Because there are no other choices, the two statements form a set of two exclusive and exhaustive hypotheses. That is, the two statements specify all possible values of parameter p .

For Example 3.3, the hypothesis statements are given in terms of the population parameter μ , the mean weight of peanuts per jar. The null hypothesis is

$$H_0: \mu = 8,$$

which is the specification for the machine to be functioning correctly. The alternative hypothesis is

$$H_1: \mu \neq 8,$$

which means the machine is malfunctioning. These statements also form a set of two exclusive and exhaustive hypotheses, even though the alternative hypothesis does not specify a single value as it did for Example 3.2.

Rules for Making Decisions

After stating the hypotheses we specify what sample results will lead to the rejection of the null hypothesis. Intuitively, sample results (summarized as sample statistics) that lead to rejection of the null hypothesis should reflect an apparent contradiction to the null hypothesis. In other words, if the sample statistics have values that are unlikely to occur if the null hypothesis is true, then we decide the null hypothesis is false. The statistical hypothesis testing procedure consists of defining sample results that appear to sufficiently contradict the null hypothesis to justify rejecting it.

In Section 2.5 we showed that a sampling distribution can be used to calculate the probability of getting values of a sample statistic from a given population. If we now define "unlikely" as some small probability, we can use the sampling distribution to determine a range of values of a sample statistic that is unlikely to occur if the null hypothesis is true. The occurrence of values in that range may then be considered grounds for rejecting that hypothesis. Statistical hypothesis testing consists of appropriately defining that region of values.

DEFINITION 3.3

The **rejection region** (also called the **critical region**) is the range of values of a sample statistic that will lead to rejection of the null hypothesis.

In Example 3.2, the null hypothesis specifies the bowl having the lower proportion of red jelly beans; hence observing a large proportion of red jelly beans would tend to contradict the null hypothesis. For now, we will arbitrarily decide that having a sample with all red jelly beans provides sufficient evidence to reject the null hypothesis. If we let Y be the number of red jelly beans, the rejection region is defined as $y = 5$.

In Example 3.3, any sample mean weight \bar{Y} not equal to 8 oz. would seem to contradict the null hypothesis. However, since some variation is expected, we would probably not want to reject the null hypothesis for values reasonably close to 8 oz. For the time being we will arbitrarily decide that a mean weight of below 7.9 or above 8.1 oz. is not "reasonably close," and we will therefore reject the null hypothesis if the mean weight of our sample occurs in this region. Thus, the rejection region for this example contains the values of $\bar{y} < 7.9$ or $\bar{y} > 8.1$.

If the value of the sample statistic falls in the rejection region, we know what decision to make. If it does not fall in the rejection region, we have a

choice of decisions. First, we could accept the null hypothesis as being true. As we will see, this decision may not be the best choice. Our other choice would be to "fail to reject" the null hypothesis. As we will see, this is not necessarily the same as accepting the null hypothesis.

Table 3.1

Results of a Hypothesis Test

The Decision	IN THE POPULATION	
	H_0 is True	H_0 is not True
H_0 is not rejected	Decision is correct	A type II error has been committed
H_0 is rejected	A type I error has been committed	Decision is correct

Possible Errors in Hypothesis Testing

In Section 3.1 we emphasized that statistical inferences based on sample data may be subject to what we called errors. Actually, it turns out that results of a hypothesis test may be subject to two distinctly different errors, which are called type I and type II errors. These errors are defined in Definitions 3.4 and 3.5 and illustrated in Table 3.1.

DEFINITION 3.4

A type I error occurs when we incorrectly reject H_0 , that is, when H_0 is actually true and our sample-based inference procedure rejects it.

DEFINITION 3.5

A type II error occurs when we incorrectly fail to reject H_0 , that is, when H_0 is actually not true, and our inference procedure fails to detect this fact.

In Example 3.2 the rejection region consisted of finding all five jelly beans in the sample to be red. Hence, the type I error occurs if all five sample jelly beans are red, the null hypothesis is rejected, and we proclaim the bowl to be bowl 1 but, in fact, bowl 2 is actually on the table. Alternatively, a type II error will occur if our sample has four or fewer red jelly beans (or one or more black jelly beans), in which case H_0 is not rejected, and we therefore proclaim that it is bowl 2, but, in fact, bowl 1 is on the table.

In Example 3.3, a type I error will occur if the machine is indeed working properly, but our sample yields a mean weight of over 8.1 or under 7.9 oz., leading to rejection of the null hypothesis and therefore an unnecessary adjustment to the machine. Alternatively, a type II error will occur if the machine is malfunctioning but the sample mean weight falls between 7.9 and 8.1 oz. In this case we fail to reject H_0 and do nothing when the machine really needs to be adjusted.

Obviously we cannot make both types of errors simultaneously, and in fact we may not make either, but the possibility does exist. In fact, we will usually

as being true.
choice would
not necessarily

never know whether any error has been committed. The only way to avoid any chance of error is not to make a decision at all, hardly a satisfactory alternative.

Probabilities of Making Errors

If we assume that we have the results of a random sample, we can use the characteristics of sampling distributions presented in Chapter 2 to calculate the probabilities of making either a type I or type II error for any specified decision rule.

DEFINITION 3.6

- α : denotes the probability of making a type I error
- β : denotes the probability of making a type II error

The ability to provide these probabilities is a key element in statistical inference, because they measure the reliability of our decisions. We will now show how to calculate these probabilities for our examples.

Calculating α for Example 3.2 The null hypothesis specifies that the probability of drawing a red jelly bean is 0.4 (bowl 2), and the null hypothesis is to be rejected with the occurrence of five red jelly beans. Then the probability of making a type I error is the probability of getting five red jelly beans in a sample of five from bowl 2. If we let Y be the number of red jelly beans in our sample of five, then

$$\alpha = P(Y = 5 \text{ when } p = 0.4).$$

The use of binomial probability distribution (Section 2.3) provides the result $\alpha = (0.4)^5 = 0.01024$. Thus the probability of incorrectly rejecting a true null hypothesis in this case is 0.01024; that is, there is approximately a 1 in 100 chance that bowl 2 will be mislabeled bowl 1 using the described decision rule.

Calculating α for Example 3.3 For this example, the null hypothesis was to be rejected if the mean weight was less than 7.9 or greater than 8.1 oz. If \bar{Y} is the sample mean weight of 16 jars, the probability of a type I error is

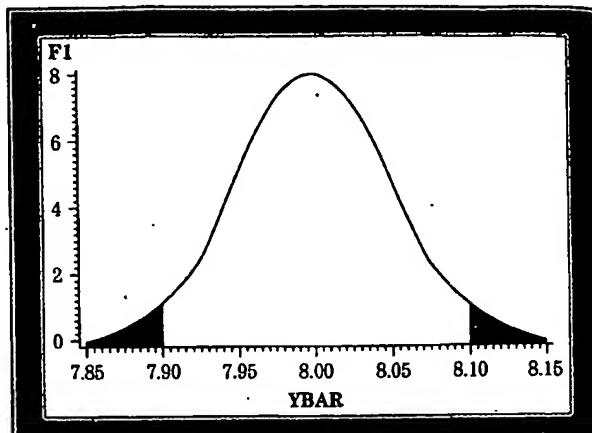
$$\alpha = P(\bar{Y} < 7.9 \text{ or } \bar{Y} > 8.1 \text{ when } \mu = 8).$$

Assume for now that we know³ that σ , the standard deviation of the population of weights, is 0.2 and that the distribution of weights is approximately normal. If the null hypothesis is true, the sampling distribution of the mean of 16 jars is normal with $\mu = 8$ and $\sigma = 0.2/\sqrt{16} = 0.05$ (see discussion on the normal distribution in Section 2.5). The probability of a type I error corresponds to the shaded area in Fig. 3.1.

³This is an assumption made here to simplify matters. In Chapter 4 we present the method required if we calculate the standard deviation from the sample data.

Figure 3.1

Rejection Region
for Sample Mean



Using the tables of the normal distribution we compute the area for each portion of the rejection region

$$P(\bar{Y} < 7.9) = P\left[Z < \frac{7.9 - 8}{0.2/\sqrt{16}}\right] = P(Z < -2.0) = 0.0228$$

and

$$P(\bar{Y} > 8.1) = P\left(Z > \frac{8.1 - 8}{0.2/\sqrt{16}}\right) = P(Z > 2.0) = 0.0228.$$

Hence

$$\alpha = 0.0228 + 0.0228 = 0.0456.$$

Thus the probability of adjusting the machine when it does not need it (using the described decision rule) is slightly less than 0.05 (or 5%).

Calculating β for Example 3.2 Having determined α for a specified decision rule, it is of interest to determine β . This probability can be readily calculated for Example 3.2. Recall that the type II error occurs if we fail to reject the null hypothesis when it is not true. For this example, this occurs if bowl 1 is on the table but we did not get the five red jelly beans required to reject the null hypothesis that bowl 2 is on the table. The probability of a type II error, which is denoted by β , is then the probability of getting four or fewer red jelly beans in a size-five sample from bowl 1. If we let Y be the number of red jelly beans in the sample, then

$$\beta = P(Y \leq 4 \text{ when } p = 0.6).$$

Using the probability rules from Section 2.2, we know that

$$P(Y \leq 4) + P(Y = 5) = 1.$$

Since $(Y = 5)$ is the complement of $(Y \leq 4)$,

$$P(Y \leq 4) = 1 - P(Y = 5).$$

Now

$$P(Y = 5) = (0.6)^5,$$

and therefore

$$\beta = 1 - (0.6)^5 = 1 - 0.07776 = 0.92224.$$

That is, the probability of making a type II error in Example 3.2 is over 92%. This value of β is unacceptably large. That is, if on the basis of this test we conclude that bowl 2 is on the table, the probability that we are wrong is 0.92!

Calculating β for Example 3.3. For Example 3.3, H_1 does not specify a single value for μ but instead includes all values of $\mu \neq 8$. Therefore, calculating the probability of the type II error requires that we examine the probability of the sample mean being outside the rejection region for every value of $\mu \neq 8$. These calculations and further discussion of β are presented later in this section where we discuss type II errors.

area for each

0228

228.

need it (using

• a specified
can be readily
s if we fail to
this occurs if
is required to
ility of a type
four or fewer
he number of

Choosing between α and β

The probability of making a type II error can be decreased by making rejection easier, which is accomplished by making the rejection region larger. For example, suppose we decide to reject H_0 if either four or five of the jelly beans are red. In this case,

$$\alpha = P(Y \geq 4 \text{ when } p = 0.4) = 0.087$$

and

$$\beta = P(Y < 4 \text{ when } p = 0.6) = 0.663.$$

Note that by changing the rejection region we succeeded in lowering β but we increased α . This will always happen if the sample size is unchanged. In fact, if by changing the rejection region α becomes unacceptably large, no satisfactory testing procedure is available for a sample of five jelly beans, a condition that often occurs when sample sizes are small (see Section 3.4). This relationship between the two types of errors prevents us from constructing a hypothesis test that has a probability of 0 for either error. In fact, the only way to ensure that $\alpha = 0$ is to never reject a hypothesis, while to ensure that $\beta = 0$ the hypothesis should always be rejected, regardless of any sample results.

Five-Step Procedure for Hypothesis Testing

In the above presentation we have shown how to determine the probability of making a type I error for some arbitrarily chosen rejection region. The more frequently used method is to specify an acceptable maximum value for α and then delineate a rejection region for a sample statistic that satisfies this value. A hypothesis test can be formally summarized as a five-step process. Briefly these steps are as follows:

Step 1: Specify H_0 , H_1 , and an acceptable level of α .

Step 2: Define a sample-based test statistic and the rejection region for the specified H_0 .

Step 3: Collect the sample data and calculate the test statistic.

Step 4: Make a decision to either reject or fail to reject H_0 . This decision will normally result in a recommendation for action.

Step 5: Interpret the results in the language of the problem. It is imperative that the results be usable by the practitioner.

We now discuss various aspects of these steps.

Step 1 consists of specifying H_0 and H_1 and a choice of a maximum acceptable value of α . This value is based on the seriousness or cost of making a type I error in the problem being considered.

DEFINITION 3.7

The **significance level** of a hypothesis test is the maximum acceptable probability of rejecting a true null hypothesis.⁴

The reason for specifying α (rather than β) for a hypothesis test is based on the premise that the type I error is of prime concern. For this reason the hypothesis statement must be set up in such a manner that the type I error is indeed the more costly. The significance level is then chosen considering the cost of making that error.

In Example 3.2, H_0 was the assertion that the bowl on the table was bowl 2. In this example interchanging H_0 and H_1 would probably not cause any major changes unless there was some extra penalty for one of the errors. Thus, we could just as easily have hypothesized that the bowl was really 1, which would have made $H_0: p = 0.6$ instead of $H_0: p = 0.4$.

In Example 3.3 we stated that the null hypothesis is $\mu = 8$. In this example the choice of the appropriate H_0 is clear. There is a definite cost if we make a type I error since this error may cause an unnecessary adjustment on a properly working machine. Of course, making a type II error is not without cost, but since we have not accepted H_0 , we are free to repeat the sampling at another time, and if the machine is indeed malfunctioning, the null hypothesis will eventually be rejected.

■ Why Do We Focus on the Type I Error?

In general, the null hypothesis is usually constructed to be that of the status quo; that is, it is the hypothesis requiring no action to be taken, no money to be spent, or in general nothing changed. This is the reason for denoting this as the null or nothing hypothesis. Since it is usually costlier to incorrectly reject the status quo than it is to do the reverse, this characterization of the null hypothesis does indeed cause the type I error to be of greater concern. In statistical hypothesis testing, the null hypothesis will invariably be stated in terms of an "equal" condition existing.

⁴Because the selection and use of the significance level is fundamental to this procedure, it is often referred to as a significance test. Although some statisticians make a minor distinction between hypothesis and significance testing, we use the two labels interchangeably.

decision will
imperative

a maximum
cost of making

acceptable

test is based
is reason the
type I error is
nsidering the

was bowl 2.
se any major
ors. Thus, we
which would

this example
t if we make
tment on a
not without
e sampling at
ll hypothesis

of the status
n, no money
for denoting
o incorrectly
zation of the
r concern. In
be stated in

edure, it is often
inction between

On the other hand, the alternative hypothesis describes conditions for which something will be done. It is the action or research hypothesis. In an experimental or research setting, the alternative hypothesis is that an established (status quo) hypothesis is to be replaced with a new one. Thus, the research hypothesis is the one we actually want to support, which is accomplished by rejecting the null hypothesis with a sufficiently low level of α such that it is unlikely that the new hypothesis will be erroneously pronounced as true.

In Example 3.2, we thought the bowl was 2 (the status quo), and would only change our mind if the sample showed significant evidence that we were wrong. In Example 3.3 the status quo is that the machine is performing correctly; hence the machine would be left alone unless the sample showed so many or so few peanuts so as to provide sufficient evidence to reject H_0 .

We can now see that it is quite important to specify an appropriate significance level. Because making the type I error is likely to have the more serious consequences, the value of α is usually chosen to be a relatively small number, and smaller in some cases than in others. That is, α must be selected so that an acceptable level of risk exists that the test will incorrectly reject the null hypothesis. Historically and traditionally, α has been chosen to have values of 0.10, 0.05, or 0.01, with 0.05 being most frequently used. These values are not sacred but do represent convenient numbers and allow the publication of statistical tables for use in hypothesis testing. We shall use these values often throughout the text. (See, however, the discussion of p values later in this section.)

Choosing α

As we saw in Example 3.2, α and β are inversely related. Unless the sample size is increased, we can reduce α only at the price of increasing β . In Example 3.2 there was little difference in the consequences of a type I or type II error; hence, the hypothesis test would probably be designed to have approximately equal levels of α and β . In Example 3.3 making the type I error will cause a costly adjustment to be made to a properly working machine, while if the type II error is committed we do not adjust the machine when needed. This error also entails some cost such as wasted peanuts or unsatisfied customers. Unless the cost of adjusting the machine is extremely high, a reasonable choice here would be to use the "standard" value of 0.05.

Some examples of problems for which one or the other type of error is more serious include the following:

- Malnutrition among young children can have serious consequences. Assume that six-year-old children should average about 10 kg in weight to be considered normal. If a sample of children from a low-income neighborhood is to be tested⁵ for subnormal weight, we would probably use $H_0: \mu = 10$ kg

⁵An alternative hypothesis that specifies values in only one direction from the null hypothesis is called a one-sided or one-tailed alternative and requires some modifications in the testing procedure. One-tailed hypothesis tests are discussed later in this section.

and $H_1: \mu < 10$ kg. Rejection of the null hypothesis implies that the children in that neighborhood are of subnormal weight, which may lead to an expanded school lunch program. A type I error would cause the initiation of an expanded school lunch program for children who do not need it, which would be an unnecessary expenditure, but would certainly do no physical harm to the children. Hence the type I error is not very serious. A type II error, on the other hand, would result in no expanded school lunch program being initiated for children who really need it. This error appears to be more serious, and a low level of β would be needed. This, of course, would indicate that a high level of α would be chosen (or a different testing principle, see Section 3.6).

- A chemist working for a major food company has developed a new formulation for instant pudding that he believes tastes better but is more expensive to make. Using a sample of taste testers and a rating scale, he tests H_0 : the mean rating of the new formulation is the same as that of the old formulation, against H_1 : the mean rating for the new pudding is larger than that of the old. A type I error would result if the hypothesis test concluded that the new pudding tastes better and it really does not. The result of this error would be marketing a product that costs more but does not taste better, probably causing the company to lose a share of the market, which would be a relatively costly error. A type II error would result in failing to market a superior pudding at this time, which could potentially result in some loss of income. Therefore, a low value for α would appear to be appropriate.
- When a drug company tests a new drug, there are two considerations that must be tested: (1) the toxicity (side effects) and (2) the effectiveness. For (1), the null hypothesis would be that the drug is toxic. This is because we would want to "prove" that it is not. For this test we would want a very small α , because a type I error would have extremely serious consequences (a significance level of 0.0001 would not be uncommon). For (2), the null hypothesis would be that the drug is not effective and a type I error would result in the drug being put on the market when it is not effective. The ramifications of this error would depend on the existing competitive drug market and the cost to both the company and society of marketing an ineffective drug.

DEFINITION 3.8

The **test statistic** is a sample statistic whose sampling distribution can be specified for both the null and alternative hypothesis case (although the sampling distribution when the alternative hypothesis is true may often be quite complex). After specifying the appropriate significance level of α , the sampling distribution of this statistic is used to define the rejection region.

it the children lead to an ex-e initiation of need it, which o no physical bus. A type II much program rs to be more ;, would indicating principle,

new formula-
re expensive
he tests H_0 :
the old for-
larger than
t concluded
esult of this
s not taste
arket, which
lt in failing
ially result
pear to be

ations that
eness. For
ecause we
ant a very
sequences
, the null
or would
he ramifi-
g market
effective

in can
ough
: may
cance
e the

DEFINITION 3.9

The rejection region comprises the values of the test statistic for which (1) the probability when the null hypothesis is true is less than or equal to the specified α and (2) probabilities when H_1 is true are greater than they are under H_0 .

In Step 2 we define the test statistic and the rejection region.

For Example 3.3 the appropriate test statistic is the sample mean. The sampling distribution of this statistic has already been used to show that the initially proposed rejection region of $\bar{Y} < 7.9$ and $\bar{Y} > 8.1$ produces a value of 0.0456 for α . If we had wanted α to be 0.05, this rejection region would appear to have been a very lucky guess! However, in most hypothesis tests it is necessary to specify α first and then use this value to delineate the rejection region. In the discussion of the significance level for Example 3.3 an appropriate level of α was chosen to be 0.05.

Remember, α is defined as

$$P(\bar{Y} \text{ falls in the rejection region when } H_0 \text{ is true}).$$

We define the rejection region by a set of boundary values, often called critical values, that are denoted by C_1 and C_2 . The probability α is then defined as

$$P(\bar{Y} < C_1 \text{ when } \mu = 8) + P(\bar{Y} > C_2 \text{ when } \mu = 8).$$

We want to find values of C_1 and C_2 so that this probability is 0.05. This is obtained by finding the C_1 and C_2 that satisfy the expression

$$\alpha = P\left[Z < \frac{C_1 - 8}{0.2/\sqrt{16}}\right] + P\left[Z > \frac{C_2 - 8}{0.2/\sqrt{16}}\right] = 0.05,$$

where Z is the standard normal variable. Because of the symmetry of the normal distribution, exactly half of the rejection region is in each tail; hence,

$$P\left[Z < \frac{C_1 - 8}{0.05}\right] = P\left[Z > \frac{C_2 - 8}{0.05}\right] = 0.025.$$

The values of C_1 and C_2 that satisfy this probability statement are found by using the standard normal table, where we find that the values of $z = -1.96$ and $z = +1.96$ satisfy our probability criteria. We use these values to solve for C_1 and C_2 in the equations $[(C_1 - 8)/0.05] = -1.96$ and $[(C_2 - 8)/0.05] = 1.96$. The solution yields $C_1 = 7.902$ and $C_2 = 8.098$; hence, the rejection region is

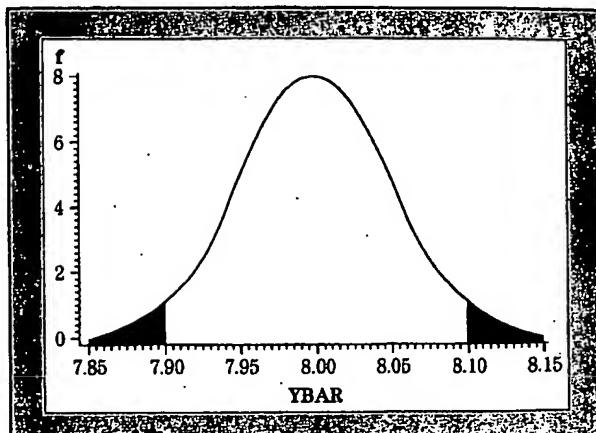
$$\bar{Y} < 7.902 \text{ or } \bar{Y} > 8.098,$$

as seen in Fig. 3.2. The rejection region of Fig. 3.2 is given in terms of the test statistic \bar{Y} , the sample mean.

It is computationally more convenient to express the rejection region in terms of a test statistic that can be compared directly to a table, such as that

Figure 3.2

Rejection Region for
0.05 Significance



of the normal distribution. In this case the test statistic is

$$\begin{aligned} Z &= \frac{\bar{Y} - \mu}{\sigma/\sqrt{n}} \\ &= \frac{\bar{Y} - 8}{0.05}, \end{aligned}$$

which has the standard normal distribution and can be compared directly with the values read from the table. Then the rejection region for this statistic is

$$z < -1.96 \text{ or } z > 1.96,$$

which can be more compactly written as $|z| > 1.96$. In other words we reject the null hypothesis if the value we calculate for Z has an absolute value (value ignoring sign) larger than 1.96.

Step 3 of the hypothesis test is to collect the sample data and compute the test statistic. (While this strict order may not be explicitly followed in practice, the sample data should not be used until the first two steps have been completed!) In Example 3.3, suppose our sample of 16 peanut jars yielded a sample mean value $\bar{y} = 7.89$. Then

$$z = (7.89 - 8)/0.05 = -2.20, \text{ or } |z| = 2.20.$$

Step 4 compares the value of the test statistic to the rejection region to make the decision. In this case we have observed that the value 2.20 is larger than 1.96 so our decision is to reject H_0 . This is often referred to as a "statistically significant" result, which means that the difference between the hypothesized value of $\mu = 8$ and the observed value of $\bar{y} = 7.89$ is large enough to be statistically significant.

In Step 5 we then conclude that the mean weight of nuts being put into jars is not the desired 8 oz. and the machine should be adjusted.

The Five Steps for Example 3.3

The hypothesis for Example 3.3 is summarized as follows:

Step 1:

$$H_0: \mu = 8$$

$$H_1: \mu \neq 8$$

$$\alpha = 0.05.$$

Step 2: The test statistic is

$$Z = \frac{\bar{Y} - 8}{0.2/\sqrt{16}}$$

whose sampling distribution is the standard normal. We specify $\alpha = 0.05$; hence we will reject H_0 if $|z| > 1.96$.

Step 3: Sample results: $n = 16$, $\bar{y} = 7.89$, $\sigma = 0.2$ (assumed);

$$z = (7.89 - 8)/[0.2/\sqrt{16}] = -2.20, \text{ hence } |z| = 2.20.$$

Step 4: $|z| > 1.96$; hence we reject H_0 .

Step 5: We conclude $\mu \neq 8$ and recommend that the machine be adjusted.

Suppose that in our initial setup of the hypothesis test we had chosen α to be 0.01 instead of 0.05. What changes? This test is summarized as follows:

Step 1:

$$H_0: \mu = 8$$

$$H_1: \mu \neq 8$$

$$\alpha = 0.01.$$

Step 2: Reject H_0 if $|z| > 2.576$.

Step 3: Sample results: $n = 16$, $\sigma = 0.2$, $\bar{y} = 7.89$;

$$z = (7.89 - 8)/0.05 = -2.20.$$

Step 4: $|z| < 2.576$; hence we fail to reject $H_0: \mu = 8$.

Step 5: We do not recommend that the machine be readjusted.

We now have a problem. We have failed to reject the null hypothesis and do nothing. However, remember that we have not proved that the machine is working perfectly. In other words, *failing to reject the null hypothesis does not mean the null hypothesis was accepted*. Instead, we are simply saying that this particular test (or experiment) does not provide sufficient evidence to have the machine adjusted at this time. In fact, in a continuing quality control program, the test will be repeated in due time.

P Values

Having to specify a significance level before making a hypothesis test seems unnecessarily restrictive because many users do not have a fixed or definite idea of what constitutes an appropriate value for α . Also it is quite difficult to do when using computers because the user would have to specify an alpha for every test being requested. Another problem with using a specified significance level is that the ultimate conclusion may be affected by very minor changes in sample statistics.

As an illustration, we observed that in Example 3.3 the sample value of 7.89 leads to rejection with $\alpha = 0.05$. However, if the sample mean had been 7.91, certainly a very similar result, the test statistic would be -1.8 , and we would not reject H_0 . In other words, the decision of whether to reject may depend on minute differences in sample results.

We also noted that with a sample mean of 7.89 we would reject H_0 with $\alpha = 0.05$ but not with $\alpha = 0.01$. The logical question then is this: What about $\alpha = 0.02$, or $\alpha = 0.03$, or ... ? This question leads to a method of reporting the results of a significance test without having to choose an exact level of significance, but instead leaves that decision to the individual who will actually act on the conclusion of the test. This method of reporting results is referred to as reporting the p value.

DEFINITION 3.10

The p value is the probability of committing a type I error if the actual sample value of the statistic is used as the boundary of the rejection region. It is therefore the smallest level of significance for which we would reject the null hypothesis with that sample. Consequently, the p value is often called the "attained" or the "empirical" significance level. It is also interpreted as an indicator of the weight of evidence against the null hypothesis.

In Example 3.3, the use of the normal table allows us to calculate the p value accurate to about four decimal places. For the sample $\bar{y} = 7.89$, this value is $P(|Z| > 2.20)$. Remembering the symmetry of the normal distribution, this is easily calculated to be $2P(Z > 2.20) = 0.0278$. This means that the management of the peanut-packing establishment can now evaluate the results of this experiment. They would reject the null hypothesis with a level of significance of 0.0278 or higher, and fail to reject it at anything lower.

Using the p value approach, Example 3.3 is summarized as follows:

Step 1:

$$H_0: \mu = 8$$

$$H_1: \mu \neq 8.$$

Step 2: Sample: $n = 16$, $\sigma = 0.2$, $\bar{y} = 7.89$;

$$z = (7.89 - 8)/0.05 = -2.20.$$

test seems or definite difficult to in alpha for significance changes in value of 7.89 been 7.91, we would depend on

ect H_0 with What about f reporting act level of will actually is referred

ie actual rejection which we ly, the p ce level. ainst the

the p value his value is tion, this is he manage- sults of this significance follows:

Step 3: $p = P(|Z| > 2.20) = 0.0278$; hence the p value is 0.0278. Therefore, we can say that the probability of observing a test statistic at least this extreme if the null hypothesis is true is 0.0278.

One feature of this approach is that the significance level need not be specified by the statistical analyst. In situations where the statistical analyst is not the same person who makes decisions, the analyst provides the p value and the decision maker determines the significance level based on the costs of making the type I error. For these reasons, many research journals now require that the results of such tests be published in this manner.

It is, in fact, actually easier for a computer program to provide p values, which are often given to three or more decimal places. However, when tests are calculated manually we must use tables. And because many tables provide for only a limited set of probabilities, p values can only be approximately determined. For example, we may only be able to state that the p value for the peanut jar example is between 0.01 and 0.05.

Note that the five steps of a significance test require that the significance level α be specified before conducting the test, while the p value is determined after the data have been collected and analyzed. Thus the use of a p value and a significance test are similar, but not strictly identical. It is, however, possible to use the p value in a significance test by specifying α in Step 1 and then altering Step 3 to read: Compute the p value and compare with the desired α . If the p value is smaller than α , reject the null hypothesis; otherwise fail to reject.

ALTERNATE DEFINITION 3.10

A p value is the probability of observing a value of the test statistic that is at least as contradictory to the null hypothesis as that computed from the sample data.

Thus the p value measures the extent to which the test statistic disagrees with the null hypothesis.

EXAMPLE 3.4

An aptitude test has been used to test the ability of fourth graders to reason quantitatively. The test is constructed so that the scores are normally distributed with a mean of 50 and standard deviation of 10. It is suspected that, with increasing exposure to computer-assisted learning, the test has become obsolete. That is, it is suspected that the mean score is no longer 50, although σ remains the same. This suspicion may be tested based on a sample of students who have been exposed to a certain amount of computer-assisted learning.

Solution The test is summarized as follows:

1.

$$H_0: \mu = 50,$$

$$H_1: \mu \neq 50.$$

2. The test is administered to a random sample of 500 fourth graders. The test statistic is

$$Z = \frac{\bar{Y} - 50}{10/\sqrt{500}}.$$

The sample yields a mean of 51.07. The test statistic has a value of

$$z = \frac{51.07 - 50}{10/\sqrt{500}} = 2.39.$$

3. The p value is computed as $2P(Z > 2.39) = 0.0168$. Because the construction of a new test is quite expensive, it may be determined that the level of significance should be less than 0.01, in which case the null hypothesis will not be rejected. However, the p value of 0.0168 may be considered sufficiently small to justify further investigation, say, by performing another experiment. ■

Type II Error and Power

In presenting the procedures for hypothesis and significance tests we have concentrated exclusively on the control over α , the probability of making the type I error. However, just because that error is the more serious one, we cannot completely ignore the type II error. There are many reasons for ascertaining the probability of that error, for example:

- The probability of making a type II error may be so large that the test may not be useful. This was the case for Example 3.2.
- Because of the trade-off between α and β , we may find that we may need to increase α in order to have a reasonable value for β .
- Sometimes we have a choice of testing procedures where we may get different values of β for a given α .

Unfortunately, calculating β is not always straightforward. Consider Example 3.3. The alternative hypothesis, $H_1: \mu \neq 8$, encompasses all values of μ not equal to 8. Hence there is a sampling distribution of the test statistic for each unique value of μ , each producing a different value for β . Therefore β must be evaluated for all values of μ contained in the alternative hypothesis, that is, all values of μ not equal to 8.

This is not really necessary. For practical purposes it is sufficient to calculate β for a few representative values of μ and use these values to plot a function representing β for all values of μ not equal to 8. A graph of β versus μ is called an "operating characteristic curve" or simply an OC curve.

To construct the OC curve for Example 3.3, we first select a few values of μ and calculate the probability of a type II error at these values. For example, consider $\mu = 7.80, 7.90, 7.95, 8.05, 8.10$, and 8.20 . Recall that for $\alpha = 0.05$ the rejection region is $\bar{Y} < 7.902$ or $\bar{Y} > 8.098$. The probability of a type II error is then the probability that \bar{Y} does not fall in the rejection region, that is, $P(7.902 \leq \bar{Y} \leq 8.098)$, which is to be calculated for each of the specific values of μ given above.

lers. The test

ie of

he construct
at the level
1 hypothesis
isidered suffi-
ing another

sts we have
f making the
e, we cannot
ascertaining

the test may

re may need

we may get

sider Exam-
ues of μ not
stic for each
re β must be
is, that is, all

cient to cal-
les to plot a
. of β versus
rve.
ew values of
or example,
r $\alpha = 0.05$
of a type II
gion, that is,
ecific values

Figure 3.3

Probability of a
Type II Error When
the Mean is 7.95

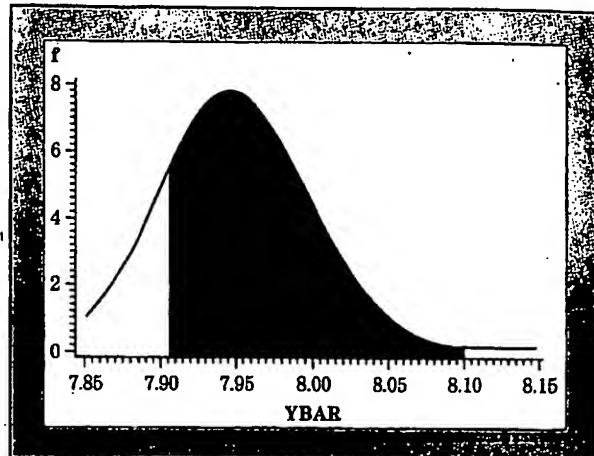


Figure 3.3 shows the sampling distribution for the mean if the population mean is 7.95 as well as the rejection region (nonshaded area) for testing the null hypothesis that $\mu = 8$. The type II error occurs when the sample mean is not in the rejection region. Therefore, as seen in the figure, the probability of a type II error when the true value of μ is 7.95 is

$$\begin{aligned}\beta &= P(7.902 \leq \bar{Y} \leq 8.098 \text{ when } \mu = 7.95) \\ &= P([(7.902 - 7.95)/0.05] \leq Z \leq [(8.098 - 7.95)/0.05]) \\ &= P(-0.96 \leq Z \leq 2.96) = 0.8300,\end{aligned}$$

obtained by using the table of the normal distribution. This probability corresponds to the shaded area in Fig. 3.3.

Similarly, the probability of a type II error when $\mu = 8.05$ is

$$\begin{aligned}\beta &= P(7.902 \leq \bar{Y} \leq 8.098 \text{ when } \mu = 8.05) \\ &= P([(7.902 - 8.05)/0.05] \leq Z \leq [(8.098 - 8.05)/0.05]) \\ &= P(-2.96 \leq Z \leq 0.96) = 0.8300.\end{aligned}$$

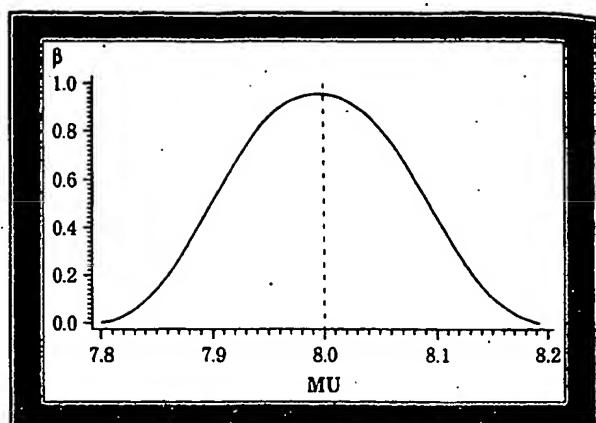
These two values of β are the same because of the symmetry of the normal distribution and also because in both cases μ is 0.05 units from the null hypothesis value. The probability of a type II error when $\mu = 7.90$, which is the same as that for $\mu = 8.10$, is calculated as

$$\begin{aligned}\beta &= P(7.902 \leq \bar{Y} \leq 8.098 \text{ when } \mu = 7.90) \\ &= P(0.04 \leq Z \leq 3.96) = 0.4840.\end{aligned}$$

In a similar manner we can obtain β for $\mu = 7.80$ and $\mu = 8.20$, which has the value 0.0207.

Figure 3.4

The OC Curve
for Example 3.3



While it is impossible to make a type II error when the true mean is equal to the value specified in the null hypothesis, β approaches $(1 - \alpha)$ as the true value of the parameter approaches that specified in the null hypothesis. The OC curve can now be constructed using these values. Figure 3.4 gives the OC curve for Example 3.3. Note that the curve is indeed symmetric and continuous. Its maximum value is $(1 - \alpha) = 0.95$ at $\mu = 8$, and it approaches zero as the true mean moves further from the H_0 value. From this OC curve we may read (at least approximately) the value of β for any value of μ we desire.

The OC curve shows the logic behind the hypothesis testing procedure as follows:

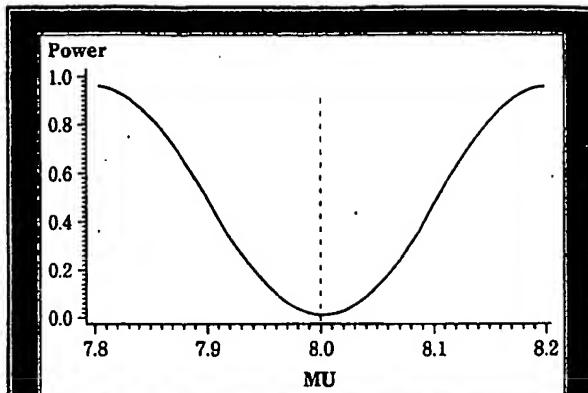
- We have controlled the probability of making the more serious type I error.
- The OC curve shows that the probability of making the type II error is larger when the difference between the true value of the mean is close to the null hypothesis value, but decreases as that difference becomes greater. In other words, the higher probabilities of failing to reject the null hypothesis occur when the null hypothesis is "almost" true, in which case the type II error may not have serious consequences.

For example, in the peanut jar problem, failing to reject simply means that we continue using the machine but also continue the sampling inspection plan. If the machine is only slightly off, continuing the operation is not likely to have very serious consequences, but since sampling inspection continues, we will have the larger probability of rejection if the machine strays very far from its target.

Power

As a practical matter we are usually more interested in the probability of not making a type II error, that is, the probability of correctly rejecting the null hypothesis when it is false.

Figure 3.5
Power Curve
for Example 3.3



DEFINITION 3.11

The power of a test is the probability of correctly rejecting the null hypothesis when it is false.

The power of a test is $(1 - \beta)$ and depends on the true value of the parameter μ . The graph of power versus all values of μ is called a **power curve**. The power curve for Example 3.3 is given in Fig. 3.5. Some features of a power curve are as follows:

- The power of the test increases and approaches unity as the true mean gets further from the null hypothesis value. This feature simply confirms that it is easier to deny a hypothesis as it gets further from the truth.
- As the true value of the population parameter approaches that of the null hypothesis, the power approaches α .
- Decreasing α while keeping the sample size fixed will produce a power curve that is everywhere lower. That is, decreasing α decreases the power.
- Increasing the sample size will produce a power curve that has a sharper "trough"; hence (except at the null hypothesis value) the power is higher everywhere. That is, increasing the sample size increases the power.

Uniformly Most Powerful Tests

Obviously high power is a desirable property of a test. If a choice of tests is available, the test with the largest power should be chosen. In certain cases, theory leads us to a test that has the largest possible power for any specified alternative hypothesis, sample size, and level of significance. Such a test is considered to be the best possible test for the hypothesis and is called a "uniformly most powerful" test. The test discussed in Example 3.3 is a uniformly most powerful test for the conditions specified in the example.

The computations involved in the construction of a power curve are not simple, and they become increasingly difficult for the applications in

subsequent chapters. Fortunately, the performance of such computations often is not necessary because virtually all of the procedures we will be using provide uniformly the most powerful tests, assuming that basic assumptions are met. We discuss these assumptions in subsequent chapters and provide some information on what the consequences may be of nonfulfillment of assumptions.

Power calculations for more complex applications can be made easier through the use of computer programs. While there is no single program that calculates power for all hypothesis tests, some programs either have the option of calculating power for specific situations or can be adapted to do so. One example using the SAS System can be found in Wright and O'Brien (1988).

One-Tailed Hypothesis Tests

In Examples 3.3 and 3.4 the alternative hypothesis simply stated that μ was not equal to the specified null hypothesis value. That is, the null hypothesis was to be rejected if the evidence showed that the population mean was either larger or smaller than that specified by the null hypothesis. For some applications we may want to reject the null hypothesis only if the value of the parameter is larger or smaller than that specified by the null hypothesis.

Solution to Example 3.1 In the example at the beginning of the chapter, we were interested in determining whether leasing office space in Atlanta costs more than that in Jacksonville. If we let μ be the mean cost per square foot of office space in Atlanta, and if we assume the standard deviation of costs is the same in both cities ($\sigma = 4.50$), we can answer the question by testing the hypothesis⁶

$$H_0: \mu = \$12.61,$$

$$H_1: \mu > \$12.61.$$

Note that the alternative hypothesis statement is now "greater than." Even though the possibility exists that the cost may be less in Atlanta than in Jacksonville, we really don't care. That is, the decision to move is to be based on the condition that the cost is higher in Atlanta. The businessman will stay in Atlanta if it costs no more to stay. The test statistic is calculated as before: $z = (13.55 - 12.61)/(4.50/6) = 1.25$. However, in this case rejection of H_0 is logical only if the value of \bar{y} is larger than that specified by H_0 , which corresponds to positive values for the test statistic z . Thus the entire rejection region is in the upper tail. A test that locates the rejection region only in one tail of the sampling distribution is known as a "one-tailed" (or one-sided) test. For this example, we will let $\alpha = 0.10$, and the rejection value is $z = 1.28$ (the

3.3

⁶To be consistent with the specification that the two hypotheses must be exhaustive, some authors will specify the null hypothesis as $\mu \leq 12.61$ for this situation. We will stay with the single-valued null hypothesis statement whether we have a one- or two-tailed alternative. We maintain the exclusive and exhaustive nature of the two hypothesis statements by stating that we do not concern ourselves with values of the parameter in the "other" tail.

MOLECULAR BIOLOGY OF THE CELL

THIRD EDITION

Bruce Alberts • Dennis Bray
Julian Lewis • Martin Raff • Keith Roberts
James D. Watson



Garland Publishing, Inc.
New York & London

also form the Fc region of the antibody, which determines what other proteins will bind to the antibody and therefore what biological properties the antibody class has. Either type of light chain (κ or λ) can be associated with any class of heavy chain, but the type of light chain does not seem to influence the properties of the antibody.

The complement system cooperates with antibodies to defend vertebrates against infection. The early components are proenzymes that circulate in the blood and are sequentially activated in an amplifying series of limited proteolytic reactions. The most important complement component is the C3 protein, which is activated by proteolytic cleavage and binds to the membrane of a microbial cell, where it helps to initiate the local assembly of the late complement components and to induce the phagocytosis of the microbial cell. The late components form large membrane attack complexes in the microbial cell membrane and thereby kill the invading microorganism.

variable region of light chain

The Fine Structure of Antibodies

Because antibodies exist in so many forms, in an unimmunized individual any one form will constitute a minute fraction of the Ig molecules in the blood. This fact presented immunochemists with a uniquely difficult problem in protein chemistry: how to obtain enough of any one antibody molecule to determine its amino acid sequence and three-dimensional structure.

The problem was solved by the discovery that the cells of a type of cancer known as **multiple myeloma** (because multiple tumors develop in the bone marrow, or myeloid tissues) secrete large amounts of a single species of antibody into the patient's blood. The antibody is homogeneous, or monoclonal, because cancer usually begins with the uncontrolled growth of a single cell, and in multiple myeloma the single cell is an antibody-secreting plasma cell. The antibody, which accumulates in the blood, is known as a **myeloma protein**.

The detailed structure of antibodies was initially determined by studying myeloma proteins from patients or from mice in which similar tumors had been purposely induced. Later it became possible to immortalize single antibody-secreting B cells by fusing them with non-antibody-secreting myeloma cells. The resultant *hybridomas* provide a ready source of monoclonal antibodies, which can be produced in unlimited amounts against any desired antigen, as discussed in Chapter 4. Today, homogeneous antibodies can also be produced in unlimited quantities by recombinant DNA technology.

Light and Heavy Chains Consist of Constant and Variable Regions^{10, 13}

Comparison of the amino acid sequences of different myeloma proteins reveals a striking feature with important genetic implications. Both light and heavy chains have a variable sequence at their amino-terminal ends but a constant sequence at their carboxyl-terminal ends. When the amino acid sequences of many different myeloma κ chains are compared, for example, the carboxyl-terminal halves are the same or show only minor differences, whereas the amino-terminal halves are all different. Thus light chains have a **constant region** about

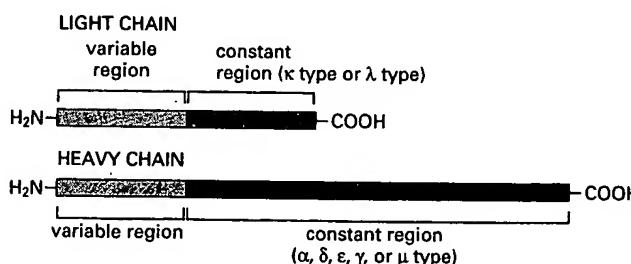


Figure 23-30 Constant and variable regions of immunoglobulin chains. Both light and heavy chains of an Ig molecule have distinct constant and variable regions.

110 ami
of the h
but the
pendin

It is
gether t
their ar
tigen-b
tant qu
and we
questio
structu

The L
Three
the Au

Scrutin
variabil
part res
parts of
These t
hyperv
tion ha
(see be
molecu
is gene
on the
as smal

The I
into I

Both lig
amino
indepe
ure 23-
main, v
stant d
and fo
bindin
the Fc

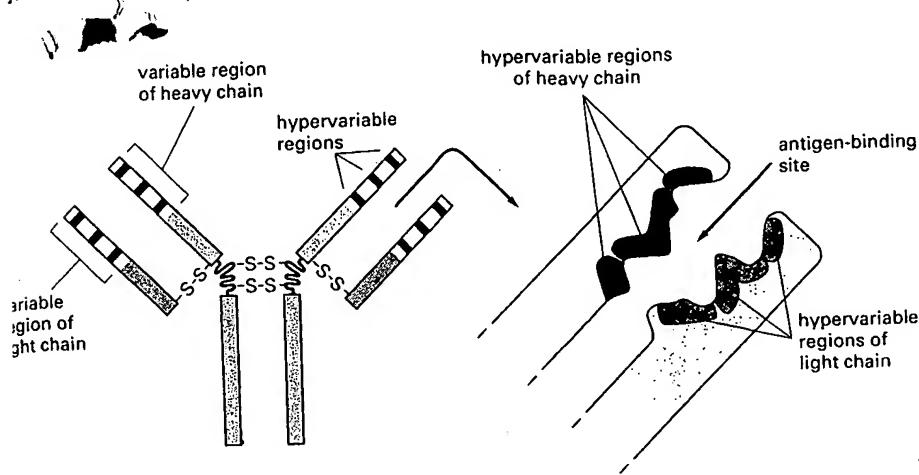


Figure 23-31 Antibody hypervariable regions. Highly schematized drawing of how the three hypervariable regions in each light and heavy chain together form the antigen-binding site of an antibody molecule. The hypervariable regions are sometimes called *complementarity-determining regions*. The actual three-dimensional structure of an antigen-binding site is shown in Figure 23-35.

110 amino acids long and a **variable region** of the same size. The variable region of the heavy chains (at their amino terminus) is also about 110 amino acids long, but the heavy-chain constant region is about 330 or 440 amino acids long, depending on the class (Figure 23-30).

It is the amino-terminal ends of the light and heavy chains that come together to form the antigen-binding site (see Figure 23-17), and the variability of their amino acid sequences provides the structural basis for the diversity of antigen-binding sites. The existence of variable and constant regions raises important questions about the genetic mechanisms that produce antibody molecules, and we consider these later. Before it became possible to investigate these genetic questions directly, other important features of antibody molecules emerged from structural studies on myeloma proteins.

The Light and Heavy Chains Each Contain Three Hypervariable Regions That Together Form the Antigen-binding Site¹⁷

Scrutiny of the amino acid sequences of a variety of Ig chains shows that the variability in the variable regions of both light and heavy chains is for the most part restricted to three small **hypervariable regions** in each chain. The remaining parts of the variable region, known as *framework regions*, are relatively constant. These findings led to the prediction that only the 5 to 10 amino acids in each hypervariable region form the antigen-binding site (Figure 23-31). This prediction has since been confirmed by x-ray diffraction studies of antibody molecules (see below). In agreement with the size of the antigen-binding site of an antibody molecule, the antigenic determinant that is specifically recognized by an antibody is generally comparably small: it can consist of fewer than 25 amino acid residues on the surface of a globular protein (see Figure 23-35), for example, and can be as small as a dinitrophenyl group (see Figure 23-7).

The Light and Heavy Chains Are Folded into Repeating Similar Domains^{10, 18}

Both light and heavy chains are made up of repeating segments—each about 110 amino acids long and each containing one intrachain disulfide bond—that fold independently to form compact functional units, or **domains**. As shown in Figure 23-32, a light chain consists of one variable (V_L) and one constant (C_L) domain, while most heavy chains consist of a variable domain (V_H) and three constant domains (C_H1 , C_H2 , and C_H3). (The μ and ϵ chains each have one variable and four constant domains.) The variable domains are responsible for antigen binding, while the constant domains of the heavy chains (excluding C_H1) form the Fc region that determines the other biological properties of the antibody.

Regulation of mesangial cell proliferation

HARALD O. SCHÖCKLMANN, STEFAN LANG, and R. BERND STERZEL

Medizinische Klinik IV, Universität Erlangen-Nürnberg, Erlangen, Germany

Regulation of mesangial cell proliferation. Regardless of the source of injury, an imbalance in the control of mesangial cell proliferation appears to play a direct role in the degree of progressive renal injury and glomerulosclerosis. Some of the regulatory mechanisms include specific soluble or non-soluble extracellular factors and a complex array of receptor-mediated signals that control the progression of the cell cycle or cell death. Understanding these regulatory processes could lead to novel therapeutic strategies to alleviate or arrest proliferative glomerular disease.

In the adult organism, normally quiescent cells exhibit increased replication rates during tumorigenesis or in the tissue repair processes following injury. In the renal glomerulus, very different types of injury are able to induce local inflammatory reactions involving resident glomerular cells. Lesions may be immune mediated, infectious, toxic, mechanical, or of other etiologies. A prominent histopathological feature of many human and experimental glomerular inflammatory diseases is cellular hyperplasia in the mesangium, which is caused by proliferation of mesangial cells (MCs) and an influx of leukocytes (Fig. 1). In this article we discuss the pathophysiological relevance of increased MC proliferation in the context of glomerular disease progression and review some of the extracellular factors and molecular mechanisms controlling MC growth.

RELEVANCE OF INCREASED MESANGIAL CELL PROLIFERATION

Pathogenetic linkage of mesangial cell hyperplasia and glomerulosclerosis

Because proliferation of MCs in the normal adult kidney is tightly regulated with a growth rate of less than 1% [1], quiescent MCs can be assumed to interact with no or few mitogens and/or to be protected by either down-regulation of receptors or the presence of growth-inhibitory factors maintaining the low proliferative activity. Regardless of the injurious mechanism, an imbalance

in the control of MC proliferation appears to play an early and crucial role in the pathogenesis of progressive glomerular injury and glomerulosclerosis. In experimental models of nephritis, MC proliferation frequently precedes and is linked to the increase of extracellular matrix (ECM) in the mesangium and glomerulosclerosis [2, 3]. For example, mice transgenic for the SV40 T antigen, which has growth-promoting functions, develop MC proliferation followed by progressive sclerosis [4], and mice transgenic for growth hormone ultimately develop severe progressive mesangial sclerosis. They show a five-fold increase in the ³H-thymidine-labeling index of glomerular cells. Interestingly, the labeling index remained high at late time points in densely sclerotic glomeruli [2], indicating that increased MC turnover can be a significant feature associated with sclerosis, both at the onset and in later stages.

Moreover, measures that reduce cell proliferation in glomerular disease models, such as treatment with heparin [5], low-protein diet [6], or neutralizing antibodies to platelet-derived growth factor (PDGF) [7], have been shown to reduce ECM expansion and sclerotic changes. Further evidence for the role of uncontrolled MC proliferation in the pathogenesis of glomerulosclerosis was provided by work of Pippin et al, who studied the effects of the pharmacological cdk2 inhibitor roscovitine on disease progression in mesangiproliferative anti-Thy1.1 nephritis of the rat [8]. The application of the purine analogue roscovitine resulted in a significant reduction of MC proliferation and glomerular cellularity in nephritic animals. Notably, the decrease of MC replication was associated with a marked reduction of mesangial ECM accumulation and less deposition of collagen type IV, laminin, and fibronectin [8].

Reconstitutive mesangial cell proliferation in glomerular repair

Persistent MC hyperplasia caused by repetitive or continuous glomerular injury is thought to precede and lead to irreversible glomerular scarring and the loss of renal function. However, like in regular wound healing, the transient increase of MC proliferation presumably reflects a physiological response required for successful

Key words: glomerulosclerosis, hyperplasia, tumorigenesis, injury, inflammation, lesions.

© 1999 by the International Society of Nephrology

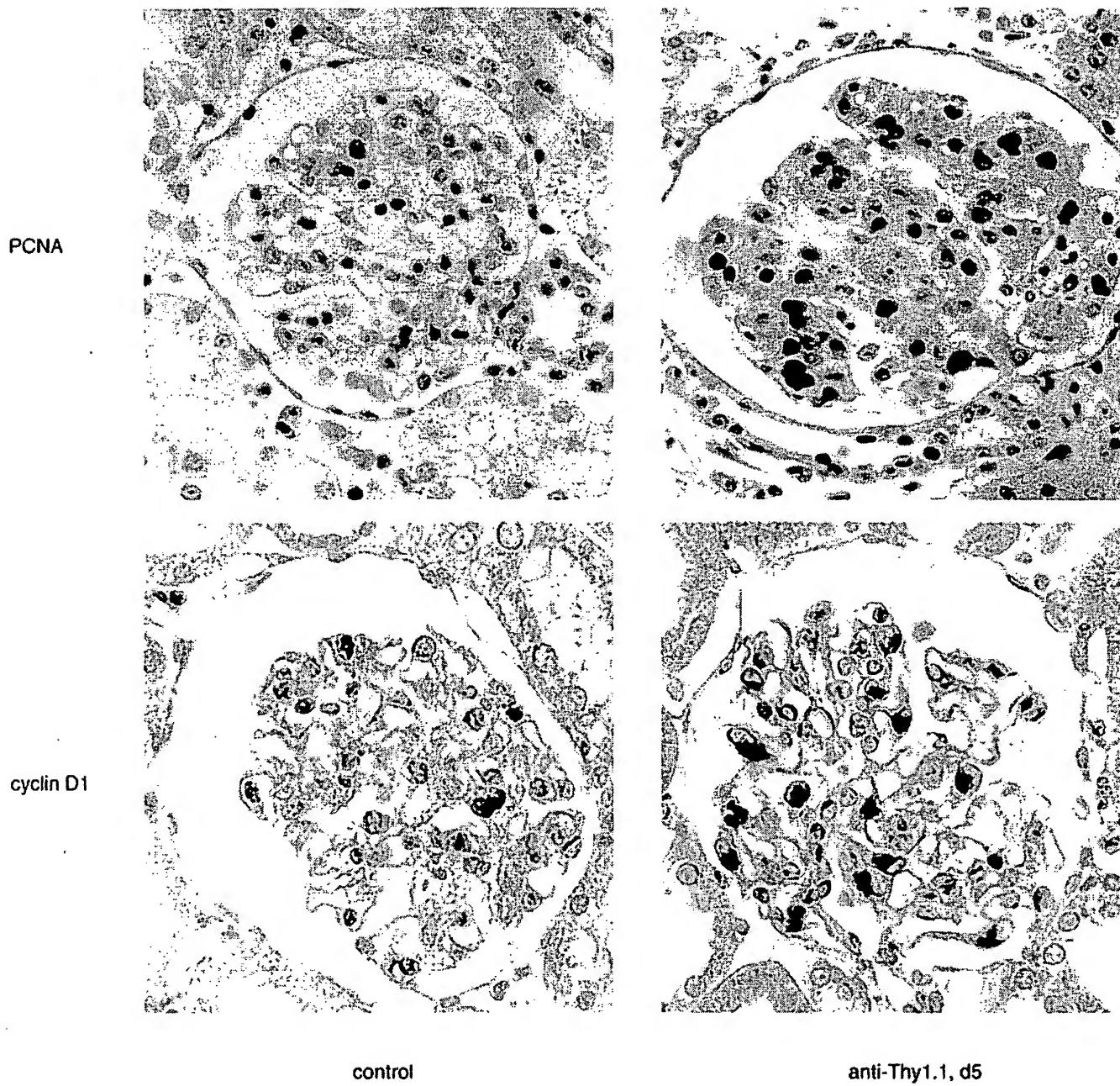


Fig. 1. Immunohistochemical detection of the proliferation marker, proliferating cell nuclear antigen (PCNA), and the G1 phase-regulatory protein cyclin D1 in rat kidney sections from normal control animal and five days after the induction of mesangiproliferative anti-Thy1.1 nephritis. PCNA-positive and cyclin D1-positive nuclei are stained black.

glomerular reconstitution and renal tissue repair. Evidence for this concept comes from observations in several types of human glomerular diseases, for example, poststreptococcal glomerulonephritis and some forms of lupus nephritis. These diseases show marked expansion of the mesangium because of MC hyperplasia, which may subsequently resolve completely once the inflammatory cause has subsided. In addition, experimental animal models of mesangiolytic injury characterized by a rapid

loss of MCs, such as anti-Thy1.1 nephritis [9] or Habu toxin glomerulopathy [10], exhibit marked proliferation of MCs leading to transient MC hyperplasia and matrix accumulation. If no repetitive insult to the mesangium is added in these animals, both diseases show spontaneous repair of the glomerular tufts within a few weeks, indicating that early and pronounced MC replication may indeed be part of the physiological reconstitutive response of glomeruli to injury, such as mesangiolysis. Thus, thera-

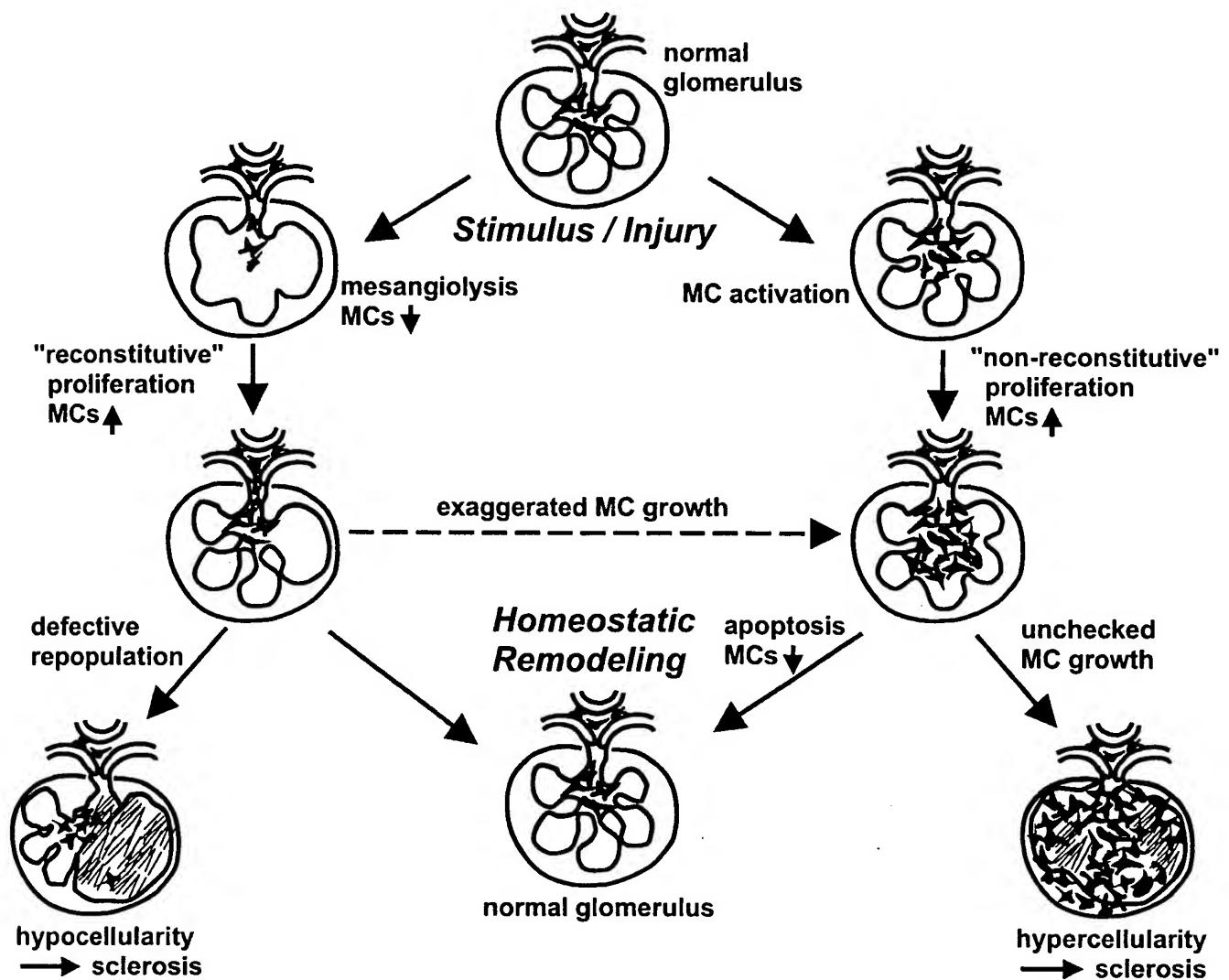


Fig. 2. Schematic illustration of two types of mesangial cell (MC) proliferation in glomerulonephritis and in homeostatic remodeling: "reconstitutive" proliferation of remaining MCs following mesangiolysis or "nonreconstitutive" MC replication after the activation of orthotopic MCs without prior cell loss. Defective homeostatic mechanisms are associated with extracellular matrix (ECM) accumulation and glomerular scarring.

peutical manipulations aiming to block MC mitogenesis could represent a double-edged sword. Although the available evidence suggests that control of overshooting MC replication is beneficial in reducing ECM accumulation, interference with reconstitutive proliferation of MCs could conceivably lead to incomplete cellular repopulation of the glomerular tuft and defective repair. Clearly, more precise markers than MC hyperplasia are needed to better characterize the dynamic nature of MC proliferation and its regulators in proliferative glomerulonephritis. This would also allow a more rational evaluation of the effects of growth-inhibitory manipulations in proliferative glomerular diseases following the loss of MCs and in chronic-progressive disease stages. Figure 2 schematically depicts two types of increased MC proliferation in response to glomerular injury. Conceptually, one

can distinguish between "reconstitutive" proliferation of the remaining MCs after mesangiolysis from "nonreconstitutive" or "orthotopic" MC replication in response to activation of intact MCs, for example, by soluble growth factors. However, it should be noted that *in vivo* these histological features may occur simultaneously, even within the same glomerulus. Currently, the methodology is insufficient to discern the nature of the proliferative processes in glomerulonephritis. This problem is aggravated in the evaluation of human glomerular diseases because of the fact that a regular kidney biopsy specimen yields but a limited number of tissue sections with rather few glomeruli. Currently, these limitations prevent thorough and meaningful studies of glomerular cell turnover in patients.

Apoptosis: A clearing mechanism balancing mesangial cell hyperplasia

When recovery of mesangioproliferative disease occurs, how is the resolution of hypercellularity achieved? Theoretically, resolution would require the reduction of the baseline regeneration or proliferation rate and/or increased removal or death of MCs. Recent research has examined cell apoptosis, a process that uses a controlled program to achieve cell death. In glomeruli, apoptotic bodies can be found in renal biopsies from patients with proliferative glomerulonephritis [11]. Baker et al and Shimizu et al demonstrated that MC apoptosis is a cell clearance mechanism counterbalancing MC division in self-limited anti-Thy1.1 glomerulonephritis, thereby contributing to the resolution of glomerular hypercellularity caused by experimentally induced MC proliferation in rats [12, 13]. In these experiments, mitotic and apoptotic cells were detected in the same glomerulus, suggesting that both processes can occur simultaneously. Although these studies imply a beneficial role of apoptosis as a mechanism leading to the reduction of MC hyperplasia and thereby contributing to the resolution of glomerular disease, other data indicate that excessive apoptosis can be harmful, leading to glomerular hypocellularity [14]. Again, these observations reflect the fact that glomerular MC numbers at any given stage during glomerular disease are determined by the rates of cell proliferation and loss. Clearly, homeostatic reconstitution of the glomerulus depends on a regulated balance between these two processes. The precise mechanisms controlling the glomerular cell turnover and net cell number in health and disease are currently incompletely understood.

MOLECULAR CONTROL OF MESANGIAL CELL CYCLE PROGRESSION

The division cycle of eukaryotic cells is controlled by a series of checkpoints and transitions in which temporal order is imposed by cyclin-dependent kinases (CDKs), acting in concert with their regulatory subunits, the cyclins (Fig. 3) [15]. Cyclin kinase inhibitors (CKIs), such as p21^{Waf-1}, p27^{Kip-1}, and members of the INK4 family of CDK inhibitors, negatively regulate the cell cycle by inhibiting the formation or activation of cyclin-CDK complexes [16]. In the past few years, considerable advances have been made in this field of research, and nephrologists are applying this knowledge to elucidate the molecular control of cell cycle progression of renal cells, including MCs. New findings on molecular mechanisms of cell cycle control were presented at this symposium and are the subject of recent reviews [16-19].

The rat anti-Thy1.1 model of mesangioproliferative glomerulonephritis has been used to study the *in vivo* expression of cell cycle regulators in glomerular cells. Shankland et al showed that the onset of MC replication

in this model is associated with significant and transient increases of cyclin A and cdk2 protein [20]. Moreover, the increase of cdk2 protein expression in the course of nephritis correlated with an increase of cdk2 activity, as measured by histone H1 kinase assay in isolated glomeruli. In normal rat glomeruli, the expression of the CKI p27^{Kip-1} was high, whereas the levels of p21^{Waf-1} were low. The onset of MC proliferation in the anti-Thy1.1 model was associated with a reduction of p27^{Kip-1} levels. The resolution of MC proliferation was associated with a return to baseline levels for p27^{Kip-1}, whereas the expression for p21^{Waf-1} further increased and remained elevated following the resolution of proliferation [20]. The relevance of the sustained p21^{Waf-1} was not fully explained, but one could speculate that p21^{Waf-1} has a functional role in the long-term resolution of mesangioproliferative disease. Currently, however, this interpretation is unproven.

Indeed, recent data have shown that p21^{Waf-1} has divergent functions. The CKI p21^{Waf-1} can act as an assembly factor of cdk4/cyclin D complexes or as an inhibitor of CDK/cyclin complexes [16, 21]. We have recently examined how soluble regulators of MC growth affect p21^{Waf-1} expression in cultured MCs. We found that PDGF causes a marked increase of p21^{Waf-1} protein in MCs [22]. The induction of p21^{Waf-1} protein by PDGF was also reported in p53-deficient as well as in normal mouse fibroblasts, supporting the interpretation of p53-independent up-regulation of p21^{Waf-1} by PDGF [23]. In other cell types, the growth-inhibitory cytokine transforming growth factor- β 1 (TGF- β 1) was found to enhance p21^{Waf-1} expression by p53-independent mechanisms [24]. However, our experiments showed that in cultured rat MCs, TGF- β 1 led to a slight reduction of the p21^{Waf-1} expression, which was induced by PDGF [22], whereas it strongly stimulated p27^{Kip-1}. These divergent findings indicate that the nature of TGF- β 1-mediated effects on cell cycle regulatory proteins differs considerably between cell types.

In our studies, we observed that increased nuclear abundance of cyclin D1 protein in MCs precedes the peak of MC hypercellularity in anti-Thy1.1 glomerulonephritis (Fig. 1), suggesting its involvement in the development of mesangioproliferative disease [22]. To further examine the relevance of the *in vivo* finding of enhanced cyclin D1 expression for MC hyperplasia, we studied the effects of soluble regulators of MC growth on cyclin D1 expression *in vitro*. In growth-arrested rat MCs in culture, mitogenic stimulation with serum or PDGF led to a rapid increase of cyclin D1 protein expression. TGF- β 1 inhibited PDGF-related induction of cyclin D1 protein. Because cyclin D1 is a key regulator of early G1-phase progression and adenovirus-mediated overexpression of cyclin D1 in MCs increased MC mitogenesis [25], we examined whether the reduction of cyclin D1 protein abundance by transfection of antisense oligonu-

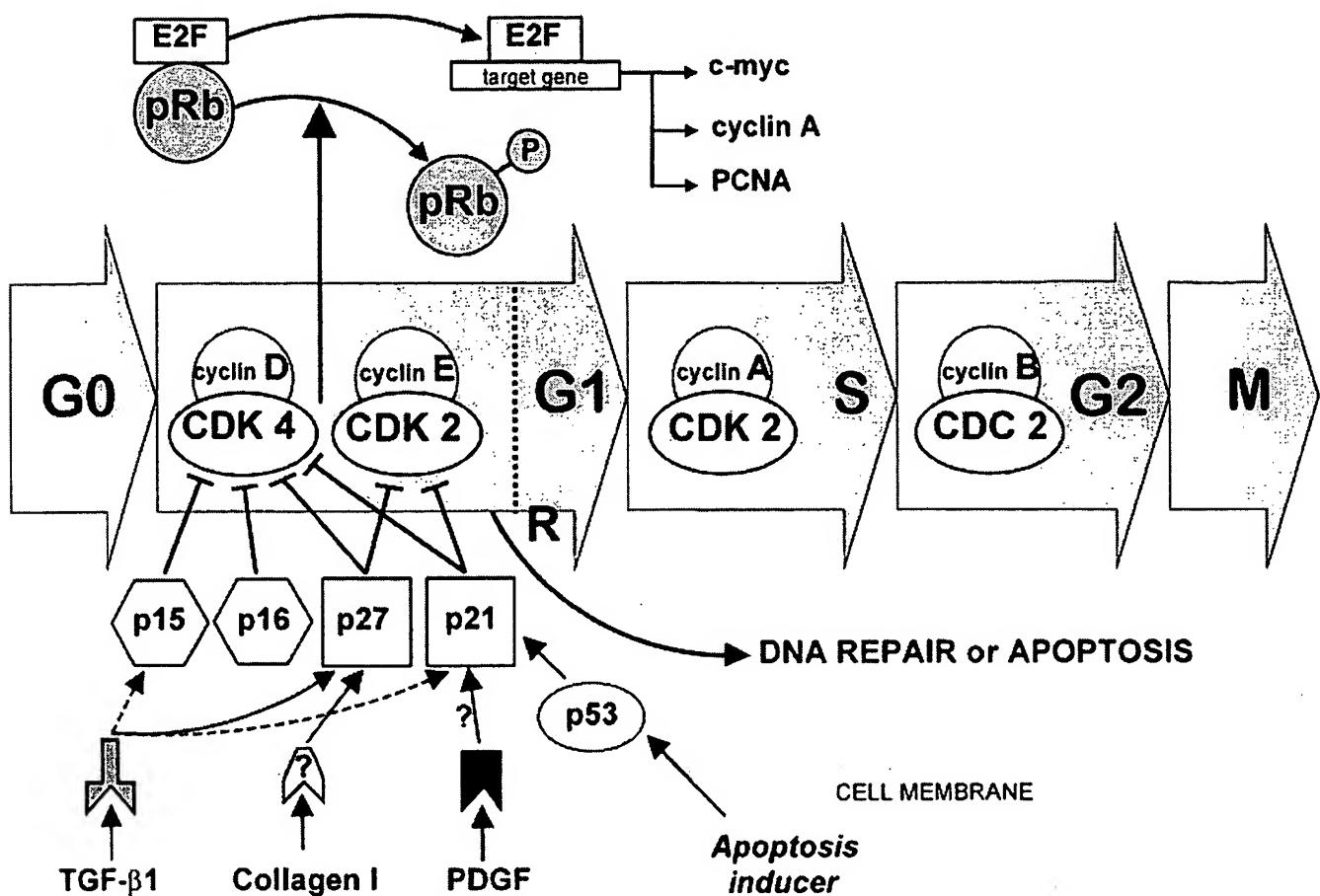


Fig. 3. Schematic diagram of the signaling pathway that mediates cell cycle progression through the G1 into the S phase. Cell cycle progression is controlled by cyclin-dependent kinases (CDK) and their regulatory subunits, the cyclins. Nuclear kinase inhibitors (for example, p15, p16, p21, and p27) modulate the enzymatic activity of cyclin/CDK complexes. External (for example, TGF- β , PDGF, collagen I) or internal regulators (for example, p53) influence the activity and/or expression of these inhibitors. The transcription factor E2F is repressed by its binding to the underphosphorylated form of the retinoblastoma gene product (pRb). Phosphorylation of pRb activates E2F, which induces the transcription of target genes essential for G1 and S phase progression. The abortion of G1 phase progression may result in apoptosis or allow for DNA repair. The restriction point (R) in the late G1 phase is where cells commit to completing the cell cycle.

cleotides (ODNs) is sufficient to inhibit cdk4 kinase activity and MC proliferation. Several studies successfully employed transfection of antisense ODNs to examine the roles of MC growth regulators and to block MC replication. In cultured MCs, proliferation could be inhibited by using antisense ODNs against the early growth response gene Egr-1 [26] or other cell cycle-associated proteins such as PCNA or Ki-67 [27]. We observed that antisense ODNs against cyclin D1 reduced the serum- or PDGF-induced protein expression of cyclin D1 to 48% or 10% of control levels, respectively [22]. These inhibitory effects correlated with diminished cdk4 activity. Subsequently, the MC proliferation caused by serum or PDGF was markedly inhibited by antisense ODNs against cyclin D1, as measured by [3 H]-thymidine uptake and MC counts. Antisense ODNs against cyclin D1 have also been shown to diminish the increase of DNA synthesis of rat MCs induced by endothelin-1 [28]. Thus, strate-

gies aiming to reduce cyclin D1 expression, for example, by transfer of antisense ODNs or by specific cyclin antagonists, might represent an effective means to inhibit MC proliferation *in vivo*.

Other studies investigated whether or not MC replication is reduced by suppressing the activity of transcription factor E2F. E2F controls the expression of several genes involved in cell cycle progression, including *c-myc*, *c-myb*, proliferating cell nuclear antigen (PCNA), and cdk2 kinase [19]. In order to inhibit E2F, decoy ODNs that contained the consensus E2F binding site sequence as a competitive inhibitor were transfected. Transfection of E2F decoy ODNs effectively reduced the proliferation of serum-stimulated MCs *in vitro* [29]. Moreover, an *in vivo* transfection of E2F decoy ODNs into rat kidneys 36 hours after the induction of anti-Thy1.1 suppressed MC proliferation by 71% [30]. These results correlated with reduced glomerular expression of PCNA [30], indicating

that decoy ODNs can inhibit the activity of E2F and may have the potential to treat proliferative forms of glomerulonephritis.

TRANSFORMING GROWTH FACTOR- β : AN AUTOCRINE INHIBITOR OF MESANGIAL CELL REPLICATION

In vitro and *in vivo* studies have identified a multitude of extracellular factors that stimulate or inhibit MC proliferation, including various soluble cytokines, autacoids, hormones, as well as insoluble ECM molecules. Many of these ligands are produced by MCs and possess autocrine growth-modulating activities [31]. Although numerous reports exist on promitogenic factors and signaling pathways in MCs, less is known about antimitogenic mechanisms that are able to block MC proliferation and might be involved in the maintenance of MC quiescence in the normal kidney and/or in the resolution of mesangioproliferative disease. TGF- β is a potent endogenous growth-inhibitory factor with the capacity to counterbalance MC mitogenesis; it is secreted by MCs and can act in an autocrine fashion [32]. Multiple studies of MCs in culture have confirmed the antimitogenic and ECM-promoting action of TGF- β [32–35]. Furthermore, in studies of experimental glomerular disease, TGF- β has been found to induce both beneficial, antimitogenic, anti-inflammatory effects [36] and harmful fibrogenesis leading to glomerular scarring [37–39]. Data from Kitamura et al demonstrated that mesangial overexpression of active TGF- β 1 by the use of MCs as a vector for gene delivery to the renal glomerulus reduced increased glomerular mitogenesis in anti-Thy1.1 nephritis, implying that TGF- β 1 acts as a suppressor of glomerular inflammation [36].

We have investigated nuclear regulatory events induced by TGF- β 1 in MCs [35]. TGF- β 1 abrogated mitogenesis of cultured MCs stimulated by PDGF, epidermal growth factor (EGF), serotonin, endothelin-1, or basic fibroblast growth factor (bFGF). By FACS analysis, we observed that the incubation of cultured MCs with TGF- β 1 blocks the progression of the MC cycle in the G1 phase of the cell cycle. One of the molecular mechanisms used by TGF- β 1 to arrest MCs in G1 phase was the significantly diminished activation of cyclin E/cdk2-complexes induced by PDGF. Subsequently, the decrease of cyclin E/cdk2 activity was reflected by less phosphorylation of the retinoblastoma tumor suppressor (pRb), a negative cell cycle regulator [35]. Deactivation of pRb via phosphorylation by G1 phase CDKs is a required step for successful G1-phase progression and S-phase entry [40]. Furthermore, in immunoprecipitation experiments we found that the presence of TGF- β 1 prevented the release of the CKI p27^{Kip1} from cyclin E/cdk2 complexes in the presence of soluble mitogens (unpublished results). This appears to be one molecular mechanism

by which cdk2 activity is negatively regulated by TGF- β 1 in MCs.

Transforming growth factor- β 1 has also been reported to cause hypertrophy of renal cells, including renal epithelial cells [41] and MCs [42, 43]. Work by Wolf et al has identified the CKI p27^{Kip1} as an important mediator of high-glucose-induced hypertrophy of MCs [44, 45]. Medium containing high d-glucose (450 mg/dl) stimulated the expression of p27^{Kip1} protein in cultured MCs. This induction of p27^{Kip1} involved activation of protein kinase C and was at least partly dependent on autoinduction of TGF- β in MCs [45]. Moreover, studies in the db/db mouse model of diabetes type II revealed an increase of p27^{Kip1} protein abundance in glomeruli of diabetic animals, which apparently was because of hyperglycemia [46]. With regard to MC growth control in glomerular disease, the available data indicate that TGF- β not only mediates the deposition of ECM but also functions as a potent autocrine inhibitor of G1-phase progression. Dependent on the type and phase of glomerular disease, the effects of TGF- β could therefore contribute to the resolution of hypercellular disease or lead to MC hypertrophy, a pathologic phenotype associated with expansion of the mesangium and, possibly, MC activation.

MODULATION OF MESANGIAL CELL GROWTH BY EXTRACELLULAR MATRIX

The ECM represents a very heterogenous source of molecular information that profoundly regulates cell behavior. Changes in the quantity and composition of the mesangial ECM can directly affect MC biology; in turn, changes in the MC activation status may result in altered ECM synthesis. Under normal conditions, mesangial ECM compounds are synthesized and degraded in a balanced manner because the integrity of glomerular matrices is strictly maintained throughout adult life. However, the loss of coordinated regulation with increase in mesangial ECM deposition occurs in the development of glomerulosclerosis, a common feature of many forms of chronic inflammatory glomerular disease.

Studies in the last decade have revealed that in addition to soluble regulator molecules, nonsoluble ECM compounds can also influence MC growth. *In vitro*, freshly seeded MCs do not only plate with much higher efficiency on collagen IV and fibronectin compared with plastic, but also show greater replication activity [47]. Thrombospondin-1, an antiadhesive matricellular glycoprotein that promotes cell rounding and division in a variety of cell types, also increases MC proliferation. This effect might be mediated in part by up-regulation of epidermal growth factor and PDGF secretion, which, in turn, can increase MC proliferation in an autocrine manner [48]. Several investigators observed dose-dependent inhibition of MC proliferation by heparan sulfate

[49, 50]. Furthermore, several proteoglycans are able to bind and sequester various cytokines, for example, TGF- β and bFGF. Local storage of such factors could represent an important regulatory function of glomerular proteoglycans. Morita et al reported that heparan sulfate proteoglycans show an enrichment of bFGF-binding domains in fibrotic lesions of the peritubular interstitium [51]. Taken together, these results support the notion that ECM molecules bind and sequester growth factors, possibly providing a local "reservoir" of regulatory proteins and presenting them to adjacent cells in a biologically more active form. This concept, however, needs experimental proof.

Several studies performed with fibroblasts have explored the molecular basis of anchorage-dependent cell growth on the level of cell cycle regulators. Zhu et al demonstrated that cell adhesion induces cyclin D1 mRNA and is required for cyclin E/cdk2 activation and subsequent phosphorylation of pRb, an effect that is possibly mediated by down-regulation of the nuclear CDK inhibitor proteins p21^{Waf1} and p27^{Kip1} [52]. Similarly, Fang et al showed that the cyclin E/cdk2 complex is activated in attached fibroblasts, but not in fibroblasts maintained in suspension [53]. These data establish a cell cycle basis to explain the combined requirement of both growth factors and ECM for anchorage-dependent growth of cells that are in transit through the G1 phase and entry into the S phase.

In addition to the need of mere cell anchorage for proliferation, the pattern of expressed adhesion molecules may also influence cell cycle progression. The expression of integrin $\beta 1C$, an alternatively spliced variant of $\beta 1$ integrin, has been described to inhibit growth by blocking cell cycle progression in the late G1 phase in fibroblasts [54] and epithelial cells [55]. Recently, integrin $\beta 1C$ was identified as an upstream regulator of the CKI p27^{Kip1}: overexpression of integrin $\beta 1C$ was associated with induction of p27^{Kip1} protein levels, inhibition of cyclin A-dependent kinase activity, and increased association of p27^{Kip1} with cyclin A [56]. Giancotti et al have examined mitogenic signaling mechanisms mediated by integrin receptors in endothelial cells and fibroblasts and could identify pathways specific for subgroups of integrins (discussed in F. Giancotti's abstract in this Symposium issue). Although most integrins are capable of activating focal adhesion kinase upon attachment to ECM, $\alpha_1\beta_1$, $\alpha_5\beta_1$, $\alpha_5\beta_3$, and $\alpha_6\beta_4$ integrins are coupled to the ras-ERK pathway via recruitment of the adaptor protein shc [57-59]. The shc adaptor links various tyrosine kinases to ras by recruiting the Grb2/SOS complex to the cell membrane [60]. In endothelial cells and keratinocytes, ligation of integrins linked to shc enabled the progression through the G1 phase in response to soluble mitogens. In contrast, ligation of other integrins resulted in growth arrest, even in the presence of soluble mitogens [58, 61].

The results of these studies indicate a direct growth-promoting function of integrin-dependent shc signaling in anchorage-dependent growth. Recently, caveolin-1 was identified as a membrane adaptor protein that links the integrin α chain to the tyrosine kinase c-fyn, which, in turn, activates and binds shc [59]. Results of our current studies have shown that these signaling molecules are also operative and regulated in cultured MCs (unpublished observations). However, the precise nuclear signaling events that mediate ECM effects to control quiescence or proliferation of MCs remain to be clarified.

Stimulated by work performed in vascular smooth muscle cells [62], we have recently tested the hypothesis that type I collagen inhibits MC proliferation by modulating cell cycle regulatory proteins of MCs in culture [63]. Although type I collagen is not expressed in glomeruli of healthy kidneys, type I collagen accumulates in the mesangial ECM in certain forms of glomerulonephritis. In the turnover of ECM, type I collagen is constantly degraded and structurally modified, for example, by proteolytic enzymes. MCs have been shown to bind to native type I collagen predominantly via $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins. As an *in vitro* system to study the effects of structurally altered ECM molecules, we examined how two structurally distinct forms of type I collagen, monomer versus polymerized fibrils, affect proliferation and expression of G1-phase regulators in MCs, using two-dimensional cell culture. The adhesion of MCs to monomer or polymerized collagen was similarly effective. However, in contrast to control substratum of plastic or monomer collagen, polymerized collagen completely prevented the serum-induced increase of DNA synthesis and MC replication. The inhibitory effect of polymerized type I collagen was characterized by the rapid down-regulation of cyclins D1 and E and the lack of serum-mediated suppression of the CKI p27^{Kip1} [63]. We speculate that polymerized type I collagen fibrils specifically regulate early $\alpha_1\beta_1$ or $\alpha_2\beta_1$ integrin signaling, which leads to inhibition of MC proliferation. In cultured MCs, polymerized type I collagen also diminished c-fos induction and the activation of various kinases involved in intracellular signaling events [64]. This study did not examine whether the ECM-driven cellular effects are regulated by integrin-mediated signals.

CONCLUSION

Taken together, the control of quiescence, proliferation, and survival of MCs greatly affects glomerular structure and function in normal or diseased kidneys. Some of the regulatory mechanisms that are operative in MCs in culture and *in vivo* have recently been clarified. They involve multiple specific soluble or nonsoluble extracellular factors, and a complex array of receptor-mediated signals that control cell cycle progression as well as

apoptosis. A better understanding of such regulatory processes is hoped to lead to more rational diagnostic as well as therapeutic approaches in patients with proliferative glomerular disease.

ACKNOWLEDGMENTS

Research data for this review were obtained with the support of the Deutsche Forschungsgemeinschaft, Bonn; Sonderforschungsbereich 263/B5.

Reprint requests to R. Bernd Sterzel, M.D., Medizinische Klinik IV, Universität Erlangen-Nürnberg, Krankenhausstr. 12, 91054 Erlangen, Germany.

REFERENCES

1. PABST R, STERZEL RB: Cell renewal of glomerular cell types in normal rats: An autoradiographic analysis. *Kidney Int* 24:626-631, 1983
2. PESCE CM, STRIKER LJ, PETEN E, ELLIOT SJ, STRIKER GE: Glomerulosclerosis at both early and late stages is associated with increased cell turnover in mice transgenic for growth hormone. *Lab Invest* 65:601-605, 1991
3. FLOEGE J, BURNS MW, ALPERS CE, YOSHIMURA A, PRITZL P, GORDON K, SEIFERT RA, BOWEN-POPE DF, COUSER WG, JOHNSON RJ: Glomerular cell proliferation and PDGF expression precede glomerulosclerosis in the remnant kidney model. *Kidney Int* 41:297-309, 1992
4. MACKAY K, STRIKER LJ, PINKERT CA, BRINSTER RL, STRIKER GE: Glomerulosclerosis and renal cysts in mice transgenic for the early region of SV40. *Kidney Int* 32:827-837, 1987
5. FLOEGE J, ENC E, YOUNG BA, COUSER WG, JOHNSON RJ: Heparin suppresses mesangial cell proliferation and matrix expansion in experimental mesangiolipid proliferative glomerulonephritis. *Kidney Int* 43:369-380, 1993
6. FUKUI M, NAKAMURA T, EBISHIMA I, NAGAOKA I, TOMINO Y, KOIDE H: Low-protein diet attenuates increased gene expression of platelet-derived growth factor and transforming growth factor- β in experimental glomerular sclerosis. *J Lab Clin Med* 121:224-234, 1993
7. JOHNSON RJ, RAINES EW, FLOEGE J, YOSHIMURA A, PRITZL P, ALPERS C, ROSS R: Inhibition of mesangial cell proliferation and matrix expansion in glomerulonephritis in the rat by antibody to platelet-derived growth factor. *J Exp Med* 175:1413-1416, 1992
8. PIPPIN JW, QU Q, MEIJER L, SHANKLAND SJ: Direct in vivo inhibition of the nuclear cell cycle cascade in experimental mesangial proliferative glomerulonephritis with Roscovitine, a novel cyclin-dependent kinase antagonist. *J Clin Invest* 100:2512-2520, 1997
9. BAGCHUS WM, HOEDEMAEKER PJ, ROZING J, BAKER WW: Glomerulonephritis induced by monoclonal anti-Thy 1.1 antibodies: A sequential histological and ultrastructural study in the rat. *Lab Invest* 55:680-687, 1986
10. CATTELL V, BRADFIELD JW: Focal mesangial proliferative glomerulonephritis in the rat caused by habu snake venom: A morphologic study. *Am J Pathol* 87:511-524, 1977
11. HARRISON DJ: Cell death in the diseased glomerulus. *Histopathology* 12:679-683, 1988
12. BAKER AJ, MOONEY A, HUGHES J, LOMBARDI D, JOHNSON RJ, SAVILL J: Mesangial cell apoptosis: The major mechanism for resolution of glomerular hypercellularity in experimental mesangial proliferative nephritis. *J Clin Invest* 94:2105-2116, 1994
13. SHIMIZU A, KITAMURA H, MASUDA Y, ISHIZAKI M, SUGISAKI Y, YAMANAKA N: Apoptosis in the repair process of experimental proliferative glomerulonephritis. *Kidney Int* 47:114-121, 1995
14. SUGIYAMA H, KASHIHARA N, MAKINO H, YAMASAKI Y, OTA A: Apoptosis in glomerular sclerosis. *Kidney Int* 49:103-111, 1996
15. SHERP CJ: d-type cyclins. *Trends Biochem Sci* 20:187-190, 1995
16. FUNK JO, GALLOWAY DA: Inhibiting CDK inhibitors: New lessons from DNA tumor viruses. *Trends Biochem Sci* 23:337-341, 1998
17. SHANKLAND SJ: Cell-cycle control and renal disease. *Kidney Int* 52:294-308, 1997
18. TERADA Y, INOSHITA S, NAKASHIMA O, KUWAHARA M, SASAKI S, MARUMO F: Cyclins and the cyclin-kinase system—their potential roles in nephrology. *Nephrol Dial Transplant* 13:1913-1916, 1998
19. DYSON N: The regulation of E2F by pRB-family proteins. *Genes Dev* 12:2245-2262, 1998
20. SHANKLAND SJ, HUGO C, COATS SR, NANGAKU M, PICHLER RH, GORDON KL, PIPPIN J, ROBERTS JM, COUSER WG, JOHNSON RJ: Changes in cell-cycle protein expression during experimental mesangial proliferative glomerulonephritis. *Kidney Int* 50:1230-1239, 1996
21. LABAER J, GARRETT MD, STEVENSON LF, SLINGERLAND JM, SANDHU C, CHOU HS, FATTAEY A, HARLOW E: New functional activities for the p21 family of CDK inhibitors. *Genes Dev* 11:847-862, 1997
22. LANG S, HARTNER A, STERZEL RB, SCHOECKLMANN HO: Requirement of cyclin D1 in mesangial cell mitogenesis. (submitted for publication)
23. MICHELI P, CHEDID M, LIN D, PIERCE JH, MERCER WE, GIVOL D: Induction of WAF1/CIP1 by a p53-independent pathway. *Cancer Res* 54:3391-3395, 1994
24. DATTO MB, LI Y, PANUS JF, HOWE DJ, XIONG Y, WANG XF: Transforming growth factor β induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proc Natl Acad Sci USA* 92:5545-5549, 1995
25. TERADA Y, YAMADA T, NAKASHIMA O, TAMAMORI M, ITO H, SASAKI S, MARUMO F: Overexpression of cell cycle inhibitors (p16INK4 and p21Cip1) and cyclin D1 using adenovirus vectors regulates proliferation of rat mesangial cells. *J Am Soc Nephrol* 8:51-60, 1997
26. HOFER G, GRIMMER C, SUKHATME VP, STERZEL RB, RUPPRECHT HD: Transcription factor Egr-1 regulates glomerular mesangial cell proliferation. *J Biol Chem* 271:28306-28310, 1996
27. MAESHIMA Y, KASHIHARA N, SUGIYAMA H, MAKINO H, OTA Z: Antisense oligonucleotides to proliferating cell nuclear antigen and Ki-67 inhibit human mesangial cell proliferation. *J Am Soc Nephrol* 7:2219-2229, 1996
28. TERADA Y, INOSHITA S, NAKASHIMA O, YAMADA T, TAMAMORI M, ITO H, SASAKI S, MARUMO F: Cyclin D1, p16, and retinoblastoma gene regulate mitogenic signaling of endothelin in rat mesangial cells. *Kidney Int* 53:76-83, 1998
29. TOMITA N, HORIUCHI M, TOMITA S, GIBBONS GH, KIM JY, BARAN D, DZAU VJ: An oligonucleotide decoy for transcription factor E2F inhibits mesangial cell proliferation in vitro. *Am J Physiol* 275:F278-F284, 1998
30. MAESHIMA Y, KASHIHARA N, YASUDA T, SUGIYAMA H, SEKIKAWA T, OKAMOTO K, KANAO K, WATANABE Y, KANWAR YS, MAKINO H: Inhibition of mesangial cell proliferation by E2F decoy oligodeoxy-nucleotide in vitro and in vivo. *J Clin Invest* 101:2589-2597, 1998
31. SCHOECKLMANN HO, RUPPRECHT HD, STERZEL RB: Mesangial cell growth control in glomerular inflammation. *Proc R Coll Phys Edinb* 26:352-373, 1996
32. SHANKLAND SJ, JOHNSON RJ: TGF- β in glomerular disease. *Miner Electrolyte Metab* 24:168-173, 1998
33. MACKAY K, STRIKER LJ, STAUFFER JW, DOI T, AGODA LY, STRIKER GE: Transforming growth factor- β : Murine glomerular receptors and responses of isolated glomerular cells. *J Clin Invest* 83:1160-1167, 1989
34. BORDER WA, OKUDA S, LANGUINO LR, RUOSLAHTI E: Transforming growth factor- β regulates production of proteoglycans by mesangial cells. *Kidney Int* 37:689-695, 1990
35. SCHOECKLMANN HO, RUPPRECHT HD, ZAUNER I, STERZEL RB: TGF- β 1-induced cell cycle arrest in renal mesangial cells involves inhibition of cyclin E-cdk 2 activation and retinoblastoma protein phosphorylation. *Kidney Int* 51:1228-1236, 1997
36. KITAMURA M, BURTON S, ENGLISH J, KAWACHI H, FINE LG: Transfer of a mutated gene encoding active transforming growth factor- β 1 suppresses mitogenesis and IL-1 response in the glomerulus. *Kidney Int* 48:1747-1757, 1995
37. BORDER WA, OKUDA S, LANGUINO LR, SPORN MB, RUOSLAHTI E: Suppression of experimental glomerulonephritis by antiserum against transforming growth factor β 1. *Nature* 346:371-374, 1990
38. BORDER WA, NOBLE NA, YAMAMOTO T, HARPER JR, YAMAGUCHI Y, PIERSCHBACHER MD, RUOSLAHTI E: Natural inhibitor of trans-

forming growth factor- β protects against scarring in experimental kidney disease. *Nature* 360:361-364, 1992

39. ISAKA Y, FUJIWARA Y, UEDA N, KANEDA Y, KAMADA T, IMAI E: Glomerulosclerosis induced by *in vivo* transfection of transforming growth factor- β or platelet-derived growth factor gene into the rat kidney. *J Clin Invest* 92:2597-2601, 1993

40. GRANA X, GARRIGA J, MAYOL X: Role of the retinoblastoma protein family, pRB, p107 and p130 in the negative control of cell growth. *Oncogene* 17:3365-3383, 1998

41. FRANCH HA, SHAY JW, ALPERN RJ, PREISIG PA: Involvement of pRB family in TGF β -dependent epithelial cell hypertrophy. *J Cell Biol* 129:245-254, 1995

42. STEPHENSON LA, HANEY LB, HUSSAINI IM, KARNS LR, GLASS WF: Regulation of smooth muscle α -actin expression and hypertrophy in cultured mesangial cells. *Kidney Int* 54:1175-1187, 1998

43. CHOI ME, KIM EG, HUANG Q, BALLERMANN BJ: Rat mesangial cell hypertrophy in response to transforming growth factor- β . *Kidney Int* 44:948-958, 1993

44. WOLF G, SHARMA K, CHEN Y, ERICKSEN M, ZIYADEH FN: High glucose-induced proliferation in mesangial cells is reversed by autocrine TGF- β . *Kidney Int* 42:647-656, 1992

45. WOLF G, SCHROEDER R, ZIYADEH FN, THAISS F, ZAHNER G, STAHL RA: High glucose stimulates expression of p27kip1 in cultured mouse mesangial cells: Relationship to hypertrophy. *Am J Physiol* 273:F348-F356, 1997

46. WOLF G, SCHROEDER R, THAISS F, ZIYADEH FN, HELMCHEN U, STAHL RA: Glomerular expression of p27kip1 in diabetic db/db mouse: Role of hyperglycemia. *Kidney Int* 53:869-879, 1998

47. SIMONSON MS, CULP LA, DUNN MJ: Rat mesangial cell-matrix interactions in culture. *Exp Cell Res* 184:484-498, 1989

48. MARINIDES GN, SUCHARD SJ, MOOKERJEE BK: Role of thrombospondin in mesangial cell growth: Possible existence of an autocrine feedback growth circuit. *Kidney Int* 46:350-357, 1994

49. CAENAZZO C, GARBISA S, CEOL M, BAGGIO B, BORSATTI A, MARCHI E, GAMBARO G: Heparin modulates proliferation and proteoglycan biosynthesis in murine mesangial cells: Molecular clues for its activity in nephropathy. *Nephrol Dial Transplant* 10:175-184, 1995

50. GROGEL GC, MARINIDES GN, HOVINGH P, HAMMOND E, LINKER A: Inhibition of rat mesangial cell growth by heparan sulfate. *Am J Physiol* 258:F259-F265, 1990

51. MORITA H, SHINZATO T, DAVID G, MIZUTANI A, HABUCHI H, FUJITA Y, ITO M, ASAI J, MAEDA K, KIMATA K: Basic fibroblast growth factor-binding domain of heparan sulfate in the human glomerulosclerosis and renal tubulointerstitial fibrosis. *Lab Invest* 71:528-535, 1994

52. ZHU X, OHTSUBO M, BOHMER RM, ROBERTS JM, ASSOIAN RK: Adhesion-dependent cell cycle progression linked to the expression of cyclin D1, activation of cyclin E-cdk2, and phosphorylation of the retinoblastoma protein. *J Cell Biol* 133:391-403, 1998

53. FANG F, OREN G, WATANABE N, HUNTER T, RUOSLAHTI E: Dependence of cyclin E-cdk2 kinase activity on cell anchorage. *Science* 271:499-502, 1996

54. MEREDITH J JR, TAKADA Y, FORNARO M, LANGUINO LR, SCHWARTZ MA: Inhibition of cell cycle progression by the alternatively spliced integrin β 1C. *Science* 269:1570-1572, 1996

55. FORNARO M, MANZOTTI M, TALLINI G, SLEAR AE, BOSARI S, RUOSLAHTI E, LANGUINO LR: β 1C integrin in epithelial cells correlates with a nonproliferative phenotype: Forced expression of β 1C inhibits its prostate epithelial cell proliferation. *Am J Pathol* 153:1079-1087, 1998

56. FORNARO M, TALLINI G, ZHENG DQ, FLANAGAN WM, MANZOTTI M, LANGUINO LR: p27 (kip1) acts as a downstream effector of and is coexpressed with the β 1C integrin in prostatic adenocarcinoma. *J Clin Invest* 103:321-329, 1999

57. MAINIERO F, PEPE A, WARY KK, SPINARDI L, MOHAMMADI M, SCHLESSINGER J, GIANCOTTI FG: Signal transduction by the α 6 β 4 integrin: Distinct β 4 subunit sites mediate recruitment of Shc/Grb2 and association with the cytoskeleton of hemidesmosomes. *EMBO J* 14:4470-4481, 1995

58. WARY KK, MAINIERO F, ISAKOFF SJ, MARCANTONIO EE, GIANCOTTI FG: The adaptor protein Shc couples a class of integrins to the control of cell cycle progression. *Cell* 87:733-743, 1996

59. WARY KK, MARIOTTI A, ZURZOLO C, GIANCOTTI FG: A requirement for caveolin-1 and associated kinase Fyn in integrin signaling and anchorage-dependent cell growth. *Cell* 94:625-634, 1998

60. PAWSON T, SCOTT JD: Signaling through scaffold, anchoring, and adaptor proteins. *Science* 278:2075-2080, 1997

61. MAINIERO F, MURGIA C, WARY KK, CURATOLA AM, PEPE A, BLUMBERG M, DER CJ, WESTWICK JK, GIANCOTTI FG: The coupling of α 6 β 4 integrin to Ras-MAP kinase pathways mediated by Shc controls keratinocyte proliferation. *EMBO J* 16:2365-2375, 1997

62. KOYAMA H, RAINES EW, BORNFELDT KE, ROBERTS JM, ROSS R: Fibrillar collagen inhibits arterial smooth muscle proliferation through regulation of Cdk2 inhibitors. *Cell* 87:1069-1078, 1996

63. SCHOECKLMANN HO, KRALEWSKI M, LANG S, STERZEL RB: Distinct structural forms of type I collagen have different effects on proliferation of glomerular mesangial cells. (submitted for publication)

64. MIRALEM T, TEMPLETON DM: Inactivation of kinase cascades in mesangial cells grown on collagen type I. *Am J Physiol* 275:F585-F594, 1998

NEW CENTRAL FAX NUMBER

Effective July 15, 2005

On July 15, 2005, the Central FAX Number will change to **571-273-8300**. This new Central FAX Number is the result of relocating the Central FAX server to the Office's Alexandria, Virginia campus.

Most facsimile-transmitted patent application related correspondence is required to be sent to the Central FAX Number. To give customers time to adjust to the new Central FAX Number, faxes sent to the old number (703-872-9306) will be routed to the new number until September 15, 2005.

After September 15, 2005, the old number will no longer be in service and **571-273-8300** will be the only facsimile number recognized for "centralized delivery".

CENTRALIZED DELIVERY POLICY: For patent related correspondence, hand carry deliveries must be made to the Customer Service Window (now located at the Randolph Building, 401 Dulany Street, Alexandria, VA 22314), and facsimile transmissions must be sent to the Central FAX number, unless an exception applies. For example, if the examiner has rejected claims in a regular U.S. patent application, and the reply to the examiner's Office action is desired to be transmitted by facsimile rather than mailed, the reply must be sent to the Central FAX Number.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

BLACK BORDERS

IMAGE CUT OFF AT TOP, BOTTOM OR SIDES

FADED TEXT OR DRAWING

BLURRED OR ILLEGIBLE TEXT OR DRAWING

SKEWED/SLANTED IMAGES

COLOR OR BLACK AND WHITE PHOTOGRAPHS

GRAY SCALE DOCUMENTS

LINES OR MARKS ON ORIGINAL DOCUMENT

REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY

OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.